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EFFECTS OF MAMMALIAN PITUITARY HORMONE PREPARATIONS ON THE CULTURED NEWT IRIS. STIMULATION OF LENS REGENERATION BY SOME THYROTROPIN PREPARATIONS

by

© ROBERT CUNY

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SUBMITTED TO THE FACULTY OF GRADUATE STUDIES AND RESEARCH
IN PARTIAL FULFILMENT OF THE REQUIREMENTS FOR THE DEGREE
OF DOCTOR OF PHILOSOPHY
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ABSTRACT

The effects of newt pituitary gland as well as mammalian pituitary hormone preparations on lens regeneration from dorsal irises of the newt (*Notophthalmus viridescens*) were studied under organ culture conditions. Irises cultured in the absence of pituitary gland or hormone preparations rarely developed up to the lens vesicle stage 4. Newt pituitary glands frequently stimulated further differentiation, with irises developing sometimes into lenses with secondary fiber cells of stage 8. Porcine adrenocorticotropin Sigma-ACTH-6002 (3 to 300 μg/ml), human thyrotropin CalBiochem-hTSH (1.6 μg/ml), bovine prolactin NIH-PRL-B4 (23 to 1600 μg/ml), bovine lutropin NIH-LH-B10 (30 to 3000 μg/ml), and bovine pituitary fibroblast growth factor CR-FGF-40002 (0.001 to 0.1 μg/ml) did not stimulate lens regeneration. The latter three preparations, as well as bovine thyrotropin Sigma-TS-10 (12 to 1400 μg/ml) inhibited depigmentation of iris epithelial cells. However, Sigma-TS-10 at high concentrations stimulated lens regeneration slightly, up to lens vesicle stage 5, similarly to bovine somatotropin NIH-GH-B18 (30 to 3000 μg/ml) and bovine follitropin NIH-FSH-B1 (30 to 2700 μg/ml). In contrast to the two previously mentioned thyrotropin preparations, ovine thyrotropin NIAMDD-oTSH-9 (1.4 μg/ml) and bovine thyrotropin NIH-TSH-B8 (3 to 3000 μg/ml) enhanced lens formation in a great proportion of cultured irises; some irises displayed large lens fiber cores characteristic of stages 8 and 9. Gamma-crystallin, a protein specific for lens fiber cells in young lenses, was detected by immunofluorescence in irises cultured with newt pituitary gland, NIAMDD-oTSH-9 or NIH-TSH-B8. The latter preparation (30 μg/ml) increased the mitotic index 5.5 times. At the lowest concentrations used in these experiments, NIAMDD-oTSH-9

(0.5 μg/ml) and NIH-TSH-B8 (0.3 μg/ml) did not significantly stimulate lens regeneration.

The effect of bovine thyrotropin NIH-TSH-B8 was also tested in cultures of dissociated iris cells. Under these conditions, NIH-TSH-B8 augmented the frequency of mitoses and stimulated formation of clusters of pigmented iris cells(iridal bodies), and depigmented cells (lentoid bodies). However, this preparation did not affect the first time of the appearance of mitotic cells, lens specific proteins, or iridal and lentoid clusters. When treatment was delayed until day 4, iris cell cultures responded very similarly to continuously treated cultures, but a delay of 15 days gave variable results. When this hormone preparation was removed on days 4 or 15 of culture, the response of the cell population resembled that of controls. Hence, this stimulator may not be involved in premitotic dedifferentiation, but may be important for mitosis and cell type conversion.

In the eye, the presence of the retina is essential for lens regeneration. An attempt was made to determine if macromolecules of the pituitary gland could also be detected in the retina. Immunofluorescence with an antiserum prepared against a crude glycoprotein extract of frog pituitary glands revealed that the antigen containing inner limiting basement membrane of the frog retina was shed five days after lentectomy, to reappear 15 days after lens removal. At five days after the operation, this antiserum stained Müller macroglial cells, which suggests that they may restore the shed membrane component.

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INTRODUCTION

1. THE REGENERATION PROBLEM IN VERTEBRATES

In the struggle for life it is an obvious advantage for an animal to be able to regenerate lost parts of its body. All vertebrates can readily regenerate lost portions of their skin, including mammalian hair, avian feathers, fish and reptilian scales, and mammalian finger nails (Goss, 1969). Only fishes and urodeles can regenerate lost appendages in the adult stage, while anurans have this ability during their larval life and lose it during metamorphosis (Goss, 1969). An exception is the African clawed frog (*Xenopus laevis*) (Deuchar, 1975), and possibly other Pipidae (Salientia) in the adult stage, which can regenerate small stumps at the amputation site. Also, if sciatic nerves are implanted at the forelimb amputation site of the adult bullfrog (*Rana catesbeiana*), the irregularly shaped limb will develop even though this species does not normally regenerate (Singer, 1951). Fishes can regenerate their tail fins, and urodeles and lizards their tails (Goss, 1969), although in the latter group regenerated tails are smaller and simplified. Newts can regenerate their lower jaws; in this case bones and teeth will be replaced but not the tongue and hyoid apparatus (Goss, 1969; Gracer, 1978). In these animals the upper jaw and snout can also regenerate if a part of the nasal capsule is left unamputated (Goss, 1969). Although differentiated nerve cells cannot proliferate, large portions of the nervous system can be regenerated in fishes and amphibians by the glial and ependymal cells, which serve as stem cells (Goss, 1969). The spinal cord can regenerate in urodeles (Windle, 1955), for example adult *Cynops pyrrhogaster* or larval *Ambystoma punctatum* (Piatt, 1955). The forebrain hemispheres can be completely regenerated in adult fishes, urodeles, and larval but not adult anurans (Goss, 1969). An exception in the latter case is again the African clawed frog (*Xenopus laevis*), which can still regenerate its forebrain after metamorphosis (Jordan, 1958). The optic tectum, however, can only be regenerated by anurans in the embryonic stage, and urodeles in the embryonic and larval stages (Goss, 1969).

This list of regenerative abilities among vertebrates could be extended, but it may suffice to point out that each phylogenetic group has its unique set of regenerative abilities preprogrammed in its genome. These abilities become apparent when a part of the body has been lost. Urodeles (Bishop, 1943), especially, have surprising regenerative abilities (Kochs, 1897). It is generally maintained (Balinsky, 1975) that organisms with a simple body plan regenerate more easily than those with a highly structured one; the newt, an animal with a complex body plan certainly contradicts this rule. It has also been claimed that regeneration approximates a recapitulation of embryonic life (Grant, 1978). This is not quite true either, since the regenerated part is replaced by the only partially dedifferentiated progeny of adult differentiated cells, which maintain normal adult physiological properties. In addition, the cellular source of the regenerated pattern may differ from the embryonic source. This is the case in lens regeneration from the iris in the newt (Wolff, 1894), as will be described below in more detail.

Some researchers have denied that lens or eye regeneration can give animals an advantage in natural selection (Wolff, 1894, 1901). Others considered that intraspecific fights, or attacks by large aquatic beetles, such as *Dysticus marginalis*, may frequently cause injuries of the eye in newts (Weismann, 1899). Trematode worms of the family Diplostomatidae, for instance *Diplostomum scheuringi* Hughes (Noble, 1954) or *D. trituri* (Kelley, 1934), preferably parasitize newt lenses (Zalokar, 1944a) or eyes (Kelley, 1934). However, other vertebrates have intraspecific fights too, are wounded by predators, or have lens- or eye-specific parasites. Natural selection acts on the level of the individual, not on the organ level. Although lens and eye regeneration may not provide decisive advantages, the enhanced general regenerative abilities may be viewed as an adaptation to colder climates, where body parts may freeze, succumb to anoxia, or injury during overwintering in inadequate resorts. The newt family with the highest number of advanced or apomorph features, the Salamandridae, also has the best regenerative abilities. This family has the widest geographic distribution, and is represented in most parts of the holarctic region. It penetrates farthest north, and climbs up highest in mountain systems. Newts cannot disperse well into tropical environments. Their metabolic rate is slower than in frogs (Noble, 1954). The reproductive success of newts is not very high in the wild (Gill, 1978), but individuals are hardy and may live up to 20 or 50 years (Noble, 1954). Hence, regeneration, rather than replacement of mutilated individuals, may be vital for the survival of newt populations.

2. STRUCTURE OF THE EYE OF THE ADULT NEWT

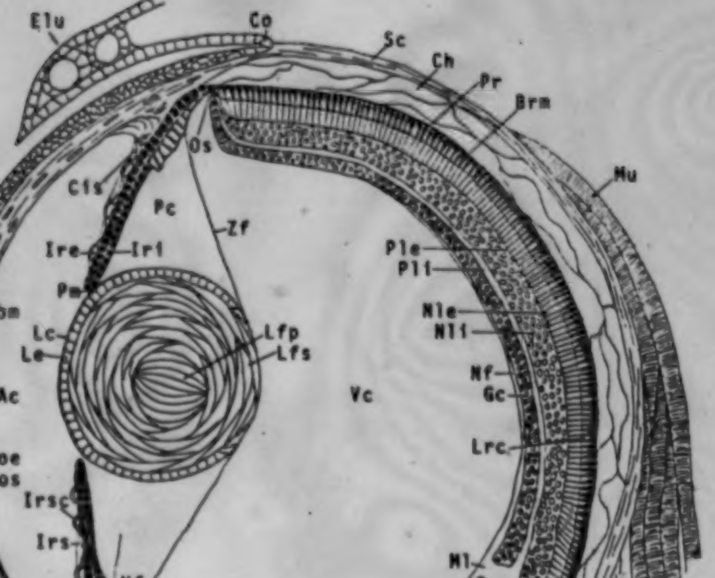
I have used the red spotted newt (*Notophthalmus viridescens*) (Rafinesque 1820)(Riley and China, 1962) for my studies on lens regeneration, and I therefore describe the structure of the adult eye of this species. Reyer (1977a) has provided a detailed description of the eye of the same species, and Rochon-Duvigneaud (1943) has made an excellent comparative study of vertebrate eyes, including urodeles.

The cornea (Figure 1) is composed of a multilayered external corneal epithelium (Coe), also called epicornea, derived from head ectoderm, and a stratified corneal stroma (Cos), the endocornea, made up of keratocytes and derived from head mesoderm. In mammals there is a corneal endothelium at the inner surface of the cornea, which produces the highly structured basement membrane of Descemet, situated between corneal stroma and corneal endothelium (Fine and Yanoff, 1979). The corneal endothelium and the membrane of Descemet seem to be missing in the adult newt eye. The epicornea is underlaid by a thin basement membrane, the membrane of Bowman (Bom), and is continuous with the single layered proximal epidermis of the eyelids at the conjunctiva (Co). In the frog *(Rana)* there is a nictitating membrane, which is absent in the newt. The eyelids (Elu, Ell) are packed with acini of the Harderian glands (Hg). At the limbus or sclerocorneal junction (Scj), the endocornea is continuous with the sclera (Sc), a fibrous connective tissue coat of the eyeball, which is of mesodermal origin. The scleral cartilage seen in the frog *(Rana)* is absent in the newt (Reyer, 1977a). Several striated retroorbital musculi recti and obliqui (Mu) attach to the proximal part of the sclera from the outside of the eyeball, and are innervated by the long ciliary nerves (Fine and Yanoff, 1979). These muscles have not yet been studies in the newt. They are involved in the bulbar eye movements. The corneal stroma and endothelium, and the sclera together are known as tunica fibrosa.

A layer of dense blood vessels, the choroid (Ch) or choriocapillaris, lines the inside of the sclera. These vessels are lined by uveal endothelial cells, either pigmented or nonpigmented, and by vascular pericytes (Fine and Yanoff, 1979); they empty into the venae bulbi, or vortex veins, which penetrate the sclera proximally and surround the optic nerve in mammals (Fine and Yanoff, 1979). No blood vessels adhere to the corneal part of the tunica fibrosa; however, the capillaries (Irsc) from the circular arteria iridica at the periphery of the iris stroma form a meshwork in the iridal and ciliary stromata. The circular arteria iridica anastomoses with bifurcations of the long posterior and anterior ciliary arteries, which penetrate the eye through the sclera close to the entrance of the optic nerve (Fine and Yanoff, 1979). The blood from the iris stroma drains into numerous ciliary lymph vessels, which themselves drain into the venous part of the choriocapillaris (Fine and Yanoff, 1979). The iris stroma (Irs) as well as the ciliary stroma (Cis) are composed of a meshwork of stromal fibroblasts, green iridophores which contain guanin pigment granules, yellow or red xanthophores which have pteridines and carotenoids as pigments, and a few black melanophores with melanin pigment. In the ciliary portion smooth muscle fibers of the tensor choroidae and protractor lentis are present, which are presumably derived from neural crest mesenchyme (Fine and Yanoff,

Figure 1. Schematic cross section of the eye of the adult red spotted newt (*Notophthalmus viridescens*).

Ac	anterior chamber
Bom	Bowman's basement membrane
Brm	Bruch's basement membrane
Ch	choroid = choriocapillaris, empties into venae bulbi
Cie	ciliary epithelium, external lamina
Cii	ciliary epithelium, internal lamina
Cis	ciliary stroma
Co	conjunctiva
Coe	corneal epithelium = epicornea
Cos	corneal stroma, part of endocornea
Ell	eye lid, lower
Elu	eye lid, upper
Gc	ganglion cell layer
Hg	Harderian gland
Ire	iris epithelium, external lamina, unspecialized dilator pupillae not shown
Iri	iris epithelium, internal lamina
Irs	iris stroma
Irsc	iris stromal capillaries of arteria iridica
Lc	lens capsule
Le	lens epithelium
Lfp	lens fibers, primary
Lfs	lens fibers, secondary
Lme	limiting membrane of neural retina, external
Lmi	limiting membrane of neural retina, internal
Lrc	layer of rods and cones, with green and red rods, cones and double cones
Ml	macula lutea = papilla nervi optici
Mu	muscles, rectus and obliqus
Nf	nerve fiber layer
Nle	nuclear layer, external, nuclei of photoreceptor cells not shown
Nli	nuclear layer, internal, with horizontal, bipolar, and amacrine cells, and nuclei of Müller cells
Ons	optic nerve sheath
Ont	optic nerve tract
Os	ora serrata, with tensor choroidae
Pc	posterior chamber
Ple	plexiform layer, external
Pli	plexiform layer, internal
Pm	pupillary margin, unspecialized sphincter iridae not shown
Pr	pigmented retina = tapetum nigrum
Sc	sclera
Scc	Schlemm's canal
Scj	sclerocorneal junction
Tm	trabecular mesh
Vc	vitreous chamber
Vf	ventral fold of iris, with protractar lentis not visible in lateral view
Zf	annular fibers


Elu
Co
Sc
Ch
Pr
Brm
Mu
Os
Cls
Pc
Zf
Ire
Irl
Pm
Bom
Lc
Le
Lfp
Lfs
Ple
Pll
Nle
Nll
Nf
Gc
Ac
Vc
Lrc
Coe
Cos
Irsc
Irs
Vf
Cle
Cll
Tm
Scc
Ml
Lme
Lmi
Ont
Ons
Hg
Ell
ScJ

1979). The latter smooth muscle is only found on the ventral iris closely associated with the embryonic choroid fissure (Reyer, 1977a), whereas the former muscle is located at the sclerocorneal junction. The intraocular fluid or aqueous humour, is resorbed via pinocytosis by the cells of the endothelial lining of the canal of Schlemm (Scc) (Pederson et al., 1978). The latter is a circular lymph vessel located at the sclerocorneal junction, and drains into the anterior ciliary veins (Fine and Yanoff, 1979). The Canal of Schlemm has not yet been studied in the newt. The angle formed by the endocornea and the ciliary stroma is filled by the trabecular mesh (Tm), a spongy tissue, which filters out debris, which might clog up the canal of Schlemm. The choroid, Schlemm's canal, the trabecular mesh, and the ciliary and iris stroma form the tunica vasculosa or uvea.

The remaining part of the eye is the tunica nervosa, made up of the iris epithelium (Ire, Iri), ciliary epithelium (Cie, Cii) and the pigmented (Pr) and neural retinae. There are three fluid-filled spaces in the interior of the eye, the anterior chamber (Ac) between the endocornea and the iris stroma, the posterior chamber (Pc) between the iris epithelium and the zonular fiber apparatus of the lens, and the vitreous chamber (Vc) between the zonular fibers (Zf) and the neural retina. The vitreous chamber is filled with an acellular matrix, mainly composed of glycosaminoglycan and some type II collagen (Bard and Abbott, 1979). The matrix is synthesized and secreted by cells of the neural retina, the ciliary epithelium and the iris epithelium (Fine and Yanoff, 1979). In bovine eyes hyalocytes in the vitreous body also contribute to the production of the vitreous matrix, especially after birth (Newsome et al., 1976). In the newt, hyalocytes may not be present, and the vitreous matrix is not sufficiently dense to form a solid vitreous body. The vitreous chamber is essentially filled by a liquid, the vitreous humour. The humour is an ultrafiltrate of the blood serum from the choroid, taken in by pinocytosis and secreted by the pigmented retinal cells (Chylack and Bellows, 1978).

In the newt the lens is almost spherical and comparatively large. It is held in the pupil by the orbiculoanterior and posterior divisions of the zonule of Zinn, which consists of zonular fibers (Zf) or suspensory ligaments (Fine and Yanoff, 1979). The zonular fibers are derived from the vitreous matrix as well as from the basement membrane material secreted by the epithelia of the iris and the ciliary regions. These fibers are attached to the iris and ciliary epithelia (Fine and Yanoff, 1979). The lens is surrounded by a thick basement membrane, the lens capsule (Lc), which throughout life is continuously synthesized and thickened by the lens epithelial cells (Fine and Yanoff, 1979). The lens epithelium (Le) surrounds the lens on its corneal side; the lens fibers reach the lens surface under the capsule on the retinal side. The primary lens fiber cells (Lfp) in the center of the lens are derived from the cells of the lens vesicle whereas the secondary fibers (Lfs) in the outer portion of the lens are derived from the equatorial region of the lens epithelium. In the newt all lens fibers meet at a vertical suture on the corneal side of the lens, and a horizontal suture on its retinal side (Reyer, 1977a). In contrast, in the frog *(Rana pipiens)* the lens fibers are essentially parallel (personal observation); in the rat they converge at a posterior and an anterior point, whereas in humans the suture is Y-shaped (Fine and Yanoff, 1979).

The iris epithelium is composed of two black pigmented laminae. Both laminae are surrounded on the outside by the iris epithelial basement membrane. On the exterior side of the basement membrane the external lamina (Ire) is covered by the iris stroma (Irs). In the center of the ring formed by the iris is the pupil, which can be widened by the dilator pupillae or made narrow by the sphincter or constrictor iridae. These are the only two smooth muscles in vertebrates which are derived from neuroectoderm (Nolte and Pointner, 1975; Fine and Yanoff, 1979). Reyer (1977a) briefly states that in the newt both of these muscles are located in the iris stroma. In the leopard frog (Nolte and Pointner, 1975) pigmented iris epithelial cells at the pupillary margin contain compact myofibrils, but the iris stroma of this species does not contain specialized contractile elements. In developing human eyes iris epithelial cells migrate out into the pupillary region of the iris stroma to form the sphincter iridae (Fine and Yanoff, 1979; Bloom and Fawcett, 1975). In addition, in humans the depigmented outer portions of the pigmented iris epithelial cells of the external lamina have assumed the muscular function of the dilator pupillae, and imbricate like shingles on a roof underneath the iris stroma (Fine and Yanoff, 1979). No cell types in the iris epithelium or iris stroma of the newt contain compact myofibrils, nor are nonpigmented cell processes apparent in iris epithelial cells of the external lamina. It appears that the pigmented iris epithelial cells in the newt have a diffuse network of contractile microfilaments which fulfil the functions of the dilator pupillae and the constrictor iridae.

In the ventral iris of the newt an inwardly directed fold (Vf) is present medially. This structure is the remnant of the choroid fissure present in the mid-ventral region of the optic cup in the embryo. In the newt the protractor lentis muscle is closely associated with this fold (Reyer, 1977a). The two laminae of the iris epithelium are continuous with the inner (Cii) and outer lamina (Cie) of the ciliary epithelium. This latter is the most important osmoregulator in the eye and contains a high sodium-potassium adenosine triphospatase activity (Rapoport, 1977). In the newt the inner lamina of the ciliary epithelium is often partially or completely depigmented (Reyer, 1977a). The ciliary epithelial laminae join the pigmented (Pr) and neural retinae at the ora serrata (Os) beneath the sclerocorneal junction (Scj). Caecae ciliaris or ciliary processes are often absent in the newt, but sometimes one or two of these folds can be found especially in the ventral region of the ciliary epithelium (Reyer, 1977a).

The cell layers of the retina are described, starting with the retinal pigmented epithelium (Pr) or tapetum nigrum on the outside, and proceding inward. The retinal pigmented epithelium is delimited by Bruch's basement membrane (Brm) (Fine and Yanoff, 1979) on its external side, facing the choroid (Ch). Internally to the pigmented epithelium is the neural retina, in which several bands can be distinguished histologically. Adjacent to the pigmented retina is the photoreceptor layer, or layer of rods and cones (Lrc). It contains red and green rod outer segments and also cone and double cone outer segments (Keefe, 1971; Dickson and Hollenberg, 1971). The nuclei and cell bodies of these neatly aligned photoreceptors are located in the outer nuclear layer

(Nle), which is separated from the layer of rods and cones by the external limiting membrane (Lme). This membrane is not a true basal membrane (Bloom and Fawcett, 1975). A thin net of nerve fibers lies inside of the outer nuclear layer, and is known as the outer plexiform layer (Ple). The inner nuclear layer follows inward. In this layer are located the horizontal cells, the amacrine cells, and the bipolar cells (Reyer, 1977a). The latter cells have large bulbar cell processes, called Landolt's clubs, which extend to the external limiting membrane (Hendricksen, 1966). These bulbar processes are often in direct contact with photoreceptor cell bodies, and they are provided with a terminal cilium (Hendricksen, 1966). In addition, the nuclei of the Müller cells, the largest glial cells of the retina are also located in the inner nuclear layer (Reyer, 1977a). Their cell processes are arranged radially within the neural retina, and span it from the internal to the external limiting membrane. These cells can be identified by Golgi's silver staining. On the inside of the inner nuclear layer lies a thick layer of nerve fibers, the inner plexiform layer (Pli), and adjacent to it the ganglion cell layer (Gc). The ganglion cell layer contains microglial astrocytes (Büssow, 1980), and the bodies of the ganglion cells, which send their axons through the overlying nerve fiber layer (Nf) to the macula lutea (Ml) or papilla nervi optici, the blind spot. There, these axons pass through the plexiform layers and penetrate out of the eye in the optic nerve tract (Ont), which is sheathed (Ons) by cells derived from the optic stalk of the embryonic eye cup. The nerve tract crosses over to the contralateral side of the brain in the optic chiasma, and connects the eye to the optic tectum in the mesencephalon. Finally, the inside of the neural retina is coated with the inner limiting basement membrane (Lmi), which may be multilayered in some areas, and is secreted by the Müller cells (Bloom and Fawcett, 1975). The fovea or area centralis (Fine and Yanoff, 1979) seems to be missing in the newt, as in the goldfish (Landreth and Agranoff, 1979). The supraretinal blood vessels on the inner limiting membrane, found in mammals (Fine and Yanoff, 1979), are absent in newt, frog and goldfish (Landreth and Agranoff, 1979). Therefore, the optic papilla, the site of entry into the eye of retinal blood vessels is absent.

3. SECONDARY EPIMORPHIC FIELDS OF THE EYE

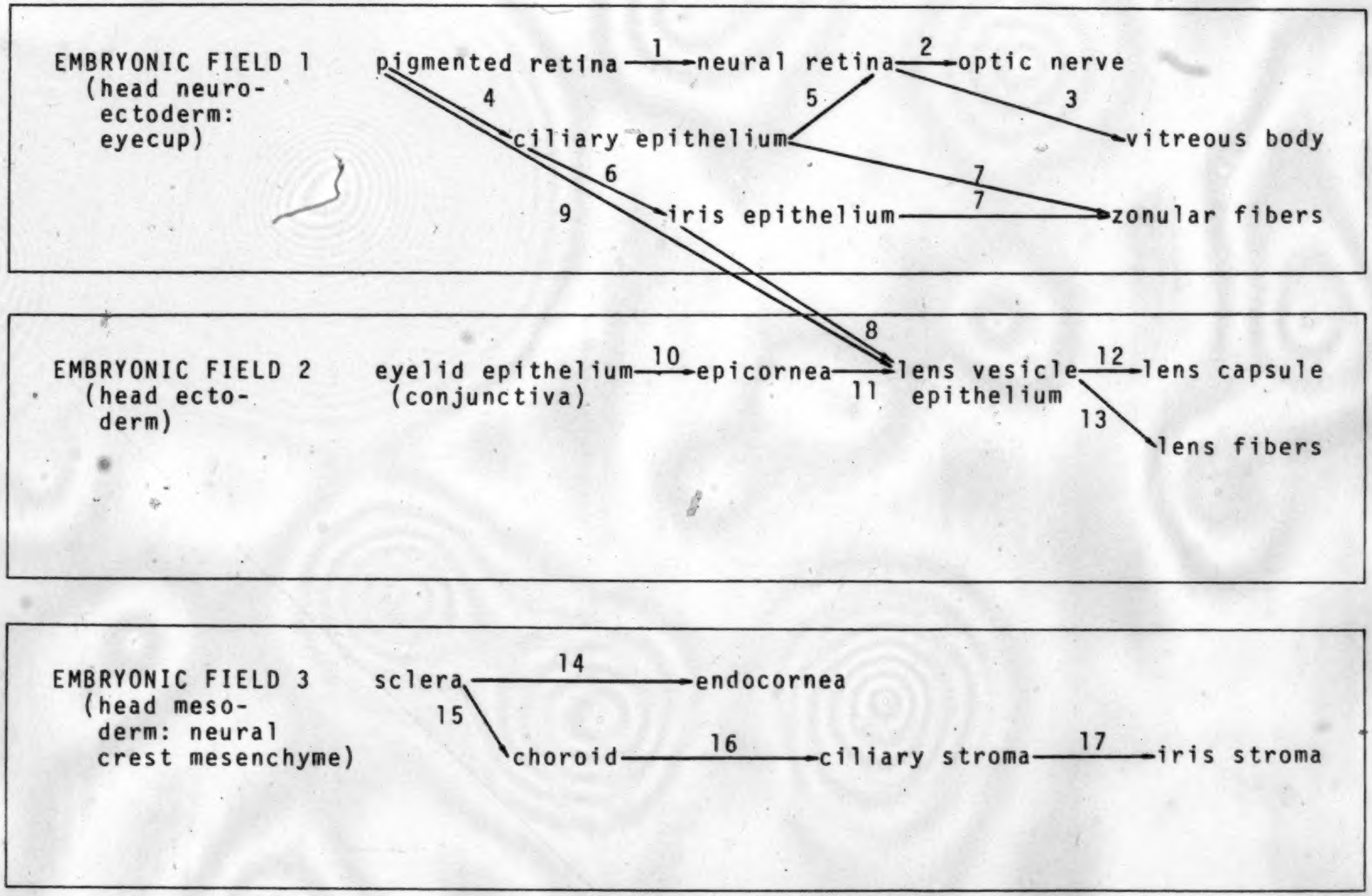
Fields (Weiss, 1969) describe the distribution of polarized positional information (Wolpert, 1971, 1977) of cells within the space limits of regenerating patterns. If the tissue of the entire field is removed, an animal cannot regenerate the lost part; however, if a small portion of the field remains, the whole field will restore itself. Morphalactic and epimorphic fields are distinguished in metazoans (Grant, 1978). During morphalactic regeneration, cells from distant regions in the body migrate into the wound, and rebuild the missing structure. Hence, no mitosis is necessary. Primary morphalactic fields encompass whole organisms, such as vertebrate embryos up to early gastrulation, adult coelenterates, and some adult planarians (Grant, 1978). Compartmentalization (García-Bellido, 1975) of the primary morphalactic field, starting at midgastrulation in vertebrate embryos, leads to further and further subdivision into secondary epimorphic fields (French, Bryant and Bryant, 1976) which can autono-

Figure 2. Ocular regeneration pathways.

Regeneration potencies in different vertebrate families of which some species have been examined by various authors. The families are members of higher phylogenetic groups (in brackets) which may rank from superorder to class level. The numbers of the regeneration pathways in the diagram refer to the families on the following list. Although the survey is comprehensive, it is not complete.

*Higginbotham (1977) claims that special fibroblasts move into the eye to form the zonular fibers.

**Van Deth (1940) observed lens regeneration in organ culture, but McKeehan (1961) could not repeat this experiment *in vivo*.


EMBRYONIC FIELD 1 (head neuro-ectoderm: eyecup)
pigmented retina
1
neural retina
2
optic nerve
3
vitreous body
4
ciliary epithelium
5
6
7
7
iris epithelium
zonular fibers
9
8
EMBRYONIC FIELD 2 (head ecto-derm)
eyelid epithelium (conjunctiva)
10
epicornea
11
lens vesicle epithelium
12
lens capsule
13
lens fibers
EMBRYONIC FIELD 3 (head meso-derm: neural crest mesenchyme)
sclera
14
endocornea
15
choroid
16
ciliary stroma
17
iris stroma

1.	Salamandridae (Caudata)	larva, adult	(Colucci, 1891; Fujita, 1913; Wachs, 1920; Stone, 1949, 1950a,b, 1956, 1959; Stone and Steinitz, 1957; Hasegawa, 1958; Mitashov, 1968, 1969a, 1970; Keefe, 1973a,b,c; Reyer, 1971b, 1977a; Levine, 1975)
	Hynobiidae (Caudata)	larva	(Ikeda, 1937)
	Pipidae (Salientia)	larva, adult	(Sologub, 1977)
	Ranidae (Salientia)	(incomplete) larva, adult	(Fujita, 1913; Sato, 1953; Rever, 1977a)
	Phasianidae (Aves)	embryo (*in vitro*)	(Coulombre and Coulombre, 1965; Reyer 1977a; Okada et al., 1979)
	Hominidae (Eutheria)	embryo (*in vitro*)	(Yasuda et al., 1978)
2.	Salamandridae (Caudata)	adult	(Mitashov, 1970; Turner and Singer, 1974)
	Pipidae (Salientia)	larva, adult	(Gaze, 1959, 1960)
	Ranidae (Salientia)	larva, adult	(Sperry, 1944; Nikitenko, 1951b)
	Bufonidae (Salientia)	larva, adult	(Sperry, 1944)
	Hylidae (Salientia)	larva, adult	(Sperry, 1944)
	Cyprinidae (Actinopterygii)	adult	(Heacock et al., 1976; Burrell et al., 1977, 1978; Wolburg, 1978; Landreth and Agranoff, 1979)
3.	Salamandridae (Caudata)	adult	(Fischel, 1897, 1900; Wachs, 1920a)
4.	Salamandridae (Caudata)	larva, adult	(Colucci, 1891; Wachs, 1920a)
	Lacertidae (Squamata)	young	(Ikeda, 1934a)
5.	Salamandridae (Caudata)	adult	(Stone, 1959; Keefe, 1973a,b,c; Reyer, 1977a)
	Ambystomatidae (Caudata)	larva, adult	(Reyer, 1971b, 1977a)
	Hynobiidae (Caudata)	embryo, larva	(Ikeda, 1935)
	Ranidae (Salientia)	(incomplete) larva, adult	(Fujita, 1913; Sato, 1953; Reyer, 1977a)
6.	Salamandridae (Caudata)	larva, adult	(Colucci, 1891; Wachs, 1920a)
7.	Salamandridae (Caudata)	adult*	(Fine and Yanoff, 1979; Higginbotham, 1977)
8.	Salamandridae (Caudata)	larva, adult	(Reyer, 1954; Stone, 1967)
	Plethodontidae (Caudata)	larva, -	(Stone, 1967)
	Cobitidae (Actinopterygii)	adult	(Sato, 1961)
	Phasianidae (Aves)	embryo**	(Van Deth, 1940; McKeehan, 1961)

9.	Salamandridae (Caudata)	larva, adult (+ *in vitro*)	(Fischel, 1900, 1903, 1921; Reyer, 1948; Sato, 1951; Stone and Steinitz, 1957; Eguchi, 1975, 1979)
	Hynobiidae (Caudata)	larva	(Ikeda, 1935)
	Phasianidae (Aves)	embryo (*in vitro*)	(Eguchi and Okada, 1973; Okada et al., 1979)
	Muridae (Eutheria)	embryo (*in vitro*)	(Eguchi, 1979)
	Hominidae (Eutheria)	embryo (*in vitro*)	(Yasuda et al., 1978)
10.	Salamandridae (Caudata)	larva, adult	(Colucci, 1891)
	Lacertidae (Squamata)	young	(Ikeda, 1934a)
11.	Hynobiidae (Caudata)	embryo, - , -	(Ikeda, 1939)
	Pipidae (Salientia)	larva -	(Freeman, 1963; Filoni et al., 1976, 1978, 1979)
12.	Salamandridae (Caudata)	adult	(Reyer, 1977a)
13.	Salamandridae (Caudata)	embryo, larva	(Reyer, 1948)
	Ambystomatidae (Caudata)	larva	(Reyer, 1977c)
	Hynobiidae (Caudata)	larva	(Ikeda, 1939; Reyer, 1954)
	Ranidae (Salientia)	larva	(Stone and Sapir, 1940; Filoni et al., 1977a)
	Cyprinodontidae (Actinopterygii)	adult	(Stone and Sapir, 1940)
	Lacertidae (Squamata)	young	(Ikeda, 1932, 1934a)
	Phasianidae (Aves)	embryo (*in vitro*)	(Piatigorsky, 1973; Beebe et al., 1979, 1980)
	Muridae (Eutheria)	embryo	(Woerdeman, 1963)
	Leporidae (Eutheria)	adult	(Stewart and Espinasse, 1959; Petit, 1963; Stone, 1965)
	Bovidae (Eutheria)	calf (*in vitro*)	(Van Venrooji et al., 1974; Courtois et al., 1981)
	Cercopithecidae (Eutheria)	adult	(Agarwal et al., 1964b)
	Hominidae (Eutheria)	adult	(Agarwal et al., 1964b)
14.	Salamandridae (Caudata)	larva, adult	(Colucci, 1891)
	Bufonidae (Salientia)	larva	(Mangold, 1931)
	Discoglossidae (Salientia)	larva	(Mangold, 1931)
	Lacertidae (Squamata)	young	(Ikeda, 1934a)
15.	Salamandridae (Caudata)	larva, adult	(Colucci, 1891)
16.	Salamandridae (Caudata)	larva, adult	(Colucci, 1891)
	Lacertidae (Squamata)	young	(Ikeda, 1934a)
17.	Salamandridae (Caudata)	larva, adult	(Colucci, 1891)

mously regulate their regeneration, but not the regeneration of their neighbouring fields. Epimorphic regeneration requires mitotic activity (Grant, 1978), because most participating cells are no longer mobile within their tissues. Typically, a blastema, a mass of actively dividing partially dedifferentiated cells, forms at the wound edge (Stocum, 1978) which has the capacity for pattern formation while its cells redifferentiate.

At the late neurula stage in anurans, for example in *Rana esculenta* and *Bufo bufo*, small fragments of tissue from the eye placode or primary eye vesicles can form whole eyes when transplanted under the skin of the belly region of a host embryo (Mangold, 1931), or when cultured *in vitro* (Perri, 1934). Thus, the presumptive eye is still an intact field at this stage. Soon thereafter, however, further compartmentalization occurs. Before the iris and retina become differentiated, the eyecup can regenerate a lens from the margin of the eyecup if the latter is experimentally separated from the head ectoderm, the usual embryonic source of the lens (Mangold, 1931; Reyer, 1948, 1950, 1954b). This ability of the embryonic eyecup is lost in later life in most vertebrates (Table 1). The adult newt (*Triturus*), however, can regenerate its eye when a large portion of it has been removed, if a little piece of sclera with adhering pigment retina in the region of the optic nerve is left in the orbit (Bonnet, 1781). Eye regeneration from one quarter of an eye takes about four months in the adult crested newt (*Triturus cristatus*), but only seven weeks in its larvae (Colucci, 1891). If the dorsal half of the eye of the newly hatched lizard (*Lacerta serpa*) is removed, the eyeball will regenerate (Ikeda, 1932). However, when cross sections of the regenerated eye are examined, one will notice that the neural retina and the lens have not been regenerated. Also, in this species if the whole iris is removed, it cannot regenerate from the ciliary epithelium (Ikeda, 1932, 1934a). Less detailed studies on eye restoration in adult toads and embryonic chickens are discussed by Mangold (1931). The hierarchy of regeneration of ocular tissues from one another resembles the embryonic sequence (Figure 2); however, several important differences suggest that regeneration requires special genetic programs, which differ at least partially from the ones used during embryonic development.

Several groups of vertebrates at the adult stage seem to be capable of lens regeneration from pieces of lens epithelium (Table 1). Lens epithelial cells constantly replace degenerating lens fiber cells throughout life. The African clawed frog (*Xenopus laevis*), and possibly other Pipidae (Anura), can regenerate the lens from the epicornea during their larval stage (Campbell and Jones, 1968). Exposure to vitreous humour seems to be required for this process (Bosco et al., 1979) *in vivo*, but not in organ culture (Campbell and Jones, 1968). The epicornea is derived from head ectoderm (Rabl, 1898), and thus stems from the same source as the primary embryonic lens. Embryonic and regeneration fields coincide in this example, since epicornea and head ectoderm are developmentally homologous parts. In adult and larval urodeles of the family Salamandridae (Caudata), and apparently in some larvae of the family Plethodontidae (Caudata) (Bishop, 1943) (Table 1), however, the lens regenerates from the dorsal iris margin (Stone, 1967). In the genus *Salamandra* the lens regenerates from the temporodorsal

Table 1. Lens regeneration capacities of selected vertebrate species.

Some authors, for example Kochs (1897), Philipeaux (1880), Grochmalicki (1908), Petersen (1921), Pasquini (1927), Pasquini and Della Monica (1929, 1930), Törö (1931, 1932), and Monroy (1939), have not critically examined, whether the lens was regenerated from iris epithelium, corneal epithelium, or lens epithelium of only partially extracted lenses. Therefore, their work could not be included here.

**Van Deth (1949) observed lens regeneration in organ culture, but McKeehan (1961) could not repeat this experiment *in vivo*.

LENS REGENERATION FROM DORSAL IRIS MARGIN

SALAMANDRIDAE (CAUDATA)		
Notophthalmus viridescens viridescens	embryo, larva, adult	(Ogawa, 1921; Stone et al., 1934; Stone and Sapir, 1940; Stone and Chace, 1941; Reyer, 1948, 1954; Stone, 1967)
Notophthalmus viridescens dorsalis	adult	(Reyer, 1954; Stone, 1967)
Notophthalmus viridescens peristriatus	adult	(Reyer, 1954; Stone, 1967)
Notophthalmus viridescens louisianensis	adult	(Stone, 1967)
Taricha granulosa granulosa	adult	(Stone, 1967)
Taricha torosa torosa	larva, adult	(Dinnean, 1942a,b; Reyer, 1954; Stone, 1967)
Taricha torosa sierrae	adult	(Reyer, 1954; Stone, 1967)
Taricha rivularis	adult	(Reyer, 1954; Stone, 1967)
Triturus vulgaris (= taeniatus)	larva, adult	(Wolff, 1895; Wachs, 1914; Sato, 1930, 1940; Zalokar, 1944a; Reyer, 1954; Stone, 1967)
Triturus helveticus	adult	(Reyer, 1954; Stone, 1967)
Triturus alpestris	larva, adult	(Sato, 1930; Reyer, 1954; Stone, 1967)
Triturus cristatus	larva, adult	(Colucci, 1891; Spemann, 1905; Wachs, 1914; Sato, 1930, 1933; Reyer, 1954; Stone, 1967)
Triturus marmoratus	adult	(Reyer, 1954; Stone, 1967)
Cynops pyrrhogaster	larva, adult	(Ogawa, 1921; Nakamura, 1935; Sato, 1940; Mikami, 1941a,b; Reyer, 1954; Stone, 1967)
Cynops ensicauda	adult	(Kojima, 1939; Reyer, 1954; Stone, 1967)
Salamandra salamandra salamandra (= maculosa)	larva, adult	(Fischel, 1897, 1900, 1903; Reinke, 1906; Wachs, 1914; Uhlenhut, 1919; Sato, 1930; Ciaccio, 1934; Reyer and Stone, 1951, 1955; Reyer, 1954; Stone, 1967)
Salamandrina perspicillata	larva	(Wachs, 1914; Stone, 1967)
Pleurodeles waltli	embryo, larva, adult	(Reyer, 1954; Vigh, 1960; Stone, 1967)
PLETHODONTIDAE (CAUDATA)		
Typhlotriton spelaeus	larva	(Stone, 1964a,b, 1967)
Eurycea lucifuga	larva	(Stone, 1967)

LENS REGENERATION FROM FUSED WHOLE IRIS MARGIN

COBITIDAE (ACTINOPTERYGII)		
Misgurnus anguillicaudatus	adult	(Sato, 1961)

LENS REGENERATION FROM VENTRAL IRIS MARGIN

PHASIANIDAE (AVES)		
Gallus gallus	embryo (no lens fibers)**	(Van Deth, 1940; McKeehan, 1961)

LENS REGENERATION FROM EPICORNEA

HYNOBIIDAE (CAUDATA)		
Hynobius unnangso	embryo	(Ikeda, 1939; Reyer, 1954)
PIPIDAE (SALIENTIA)		
Xenopus laevis	larva	(Overton, 1965; Campbell and Jones, 1968; Waggoner, 1973; Bosco et al., 1979)

8

LENS REGENERATION FROM LENS EPITHELIUM

SALAMANDRIDAE (CAUDATA)		
Notophthalmus viridescens viridescens	embryo, larva	(Reyer, 1948)
AMBYSTOMATIDAE (CAUDATA)		
Ambystoma punctatum maculatum	embryo, larva, adult	(Stone and Dinnean, 1940; Stone and Sapir, 1940; Reyer, 1962, 1977c)
Ambystoma tigrinum tigrinum	larva	(Stone and Sapir, 1940)
HYNOBIIDAE (CAUDATA)		
Hynobius unnangso	larva	(Ikeda, 1934b; Reyer, 1954)
RANIDAE (SALIENTIA)		
Rana esculenta	larva	(Filoni et al., 1977a)
Rana temporaria (=fusca)	larva	(Woerdeman, 1922; Stone and Sapir, 1940)
CYPRINODONTIDAE (ACTINOPTERYGII)		
Fundulus heteroclitus	adult	(Stone and Sapir, 1940)
LACERTIDAE (SQUAMATA)		
Lacerta serpa	young	(Ikeda, 1932, 1934a)
Lacerta vivipara	young	(Ikeda, 1932, 1934a)
PHASIANIDAE (AVES)		
Gallus gallus	embryo (*in vitro*)	(Piatigorsky, 1973; Beebe et al., 1979, 1980)
MURIDAE (RODENTIA)		
Rattus rattus	embryo	(Woerdeman, 1963)
LEPORIDAE (LAGOMORPHA)		
Oryctolagus cuniculus	adult	(Binder et al., 1961, 1962; Petit, 1963; Agarwal et al., 1964a,c; Stone, 1965)
BOVIDAE (ARTIODACTYLA)		
Bos taurus	calf (*in vitro*)	(Van Venrooji et al., 1974; Courtois et al., 1981)
CERCOPITHECIDAE (PRIMATES)		
Macaca mulatta	adult	(Agarwal et al., 1964b)
HOMINIDAE (PRIMATES)		
Homo sapiens	adult	(Agarwal et al., 1964b)

ABSENCE OF LENS REGENERATION FROM LENS EPITHELIUM

SALAMANDRIDAE (CAUDATA)		
Notophthalmus viridescens viridescens	adult	(Stone and Sapir, 1940; Stone, 1943)

ABSENCE OF LENS REGENERATION FROM EITHER IRIS OR EPICORNEA

PLETHODONTIDAE (CAUDATA)		
Typhlotriton speleus	adult	(Stone, 1964a,b; Stone, 1967)
Eurycea ensicauda melanopleura	adult	(Stone, 1967)
Ensatina escholtzii picta	adult	(Stone, 1967)
Desmognatus auriculatus	adult	(Stone, 1967)
Batrachoseps attenuatus attenuatus	adult	(Stone, 1967)

AMBYSTOMATIDAE (CAUDATA)		
Ambystoma punctatum maculatum	embryo, larva, adult	(Stone and Dinnean, 1940; Reyer, 1954, 1962; Stone, 1967)
Ambystoma tigrinum tigrinum	larva	(Stone and Dinnean, 1940; Reyer, 1962; Stone, 1967)
Ambystoma mexicanum	embryo, larva	(Wachs, 1914; Manuilowa and Kislow, 1934; Reyer, 1954; Stone, 1967)
Ambystoma opacum	larva	(Stone, 1967)
HYNOBIIDAE (CAUDATA)		
Hynobius unnangso	larva	(Ikeda, 1934b,c, 1935, 1936b; Reyer, 1954)
Hynobius nebulosus	larva	(Ikeda and Amatatu, 1941; Reyer, 1954)
Hynobius nigricans	larva	(Reyer, 1954)
RANIDAE (SALIENTIA)		
Rana temporaria (=fusca)	embryo, larva, adult	(Spemann, 1912; Woerdeman, 1922; Reyer, 1954; Simirskii, 1979)
Rana esculenta	embryo, larva, adult	(Spemann, 1912; Reyer, 1954; Filoni et al., 1976)
Rana sylvatica	larva	(Stone and Sapir, 1940; Reyer, 1954)
Rana pipiens	larva, adult	(Stone and Sapir, 1940; personal observation)
Rana clamitans	larva	(Stone and Sapir, 1940)
Rana rugosa	adult	(Okada, 1939; Reyer, 1954)
Rana japonica	adult	(Okada, 1939; Reyer, 1954)
Rana nigromaculata	larva, adult	(Okada, 1939, 1943; Reyer, 1954)
Rana arvalis	adult	(Reyer, 1954)
Rana dalmatina	larva	(Cioni et al., 1979)
BUFONIDAE (SALIENTIA)		
Bufo bufo	adult	(Reyer, 1954)
Bufo viridis	adult	(Reyer, 1954)
PIPIDAE (SALIENTIA)		
Xenopus laevis	adult	(Campbell and Jones, 1968)
DISCOGLOSSIDAE (SALIENTIA)		
Discoglossus pictus	larva, adult	(Reyer, 1954; Filoni et al., 1977b)
Bombinator pachypus	embryo, larva	(Spemann, 1912; Mangold, 1931)
Bombina bombina	larva, adult	(Nikitenko, 1937; Reyer, 1954)
HYLIDAE (SALIENTIA)		
Hyla arborea	embryo, larva, adult	(Ekman, 1914; Okada, 1939; Reyer, 1954)
PELOBATIDAE (SALIENTIA)		
Pelobates fuscus	embryo, larva, adult	(Reyer, 1954)
CYPRINODONTIDAE (ACTINOPTERYGII)		
Fundulus heteroclitus	embryo, larva, adult	(Stockard, 1910; Stone and Sapir, 1940; Sato, 1961)
Phoxinus laevis	adult	(Alberti, 1922)
CYPRINIDAE (ACTINOPTERYGII)		
Carassius auratus	adult	(Sato, 1961)
Cyprinus carpio	adult	(Sato, 1961)
Oryzias latipes	adult	(Sato, 1961)

SALMONIDAE (ACTINOPTERYGII)				
Salmo salar			adult	(Baurmann, 1922; Alberti, 1922)
Trutta fabio		larva		(Alberti, 1922)
Trutta iridea		larva		(Alberti, 1922)
LACERTIDAE (SQUAMATA)				
Lacerta saepa		young		(Ikeda, 1932, 1934a)
Lacerta vivipara		young		(Ikeda, 1932, 1934a)
SCINCIDAE (SQUAMATA)				
Eumeces latisculatus			adult	(Mangold, 1931)
PHASIANIDAE (AVES)				
Gallus gallus	embryo**		adult	(Van Deth, 1940; McKeehan, 1961)
MURIDAE (RODENTIA)				
Rattus rattus	embryo			(Woerdeman, 1963)
LEPORIDAE (LAGOMORPHA)				
Oryctolagus cuniculus			adult	(Agarwal et al., 1964a; Stone, 1965)
CERCOPITHECIDAE (PRIMATES)				
Macaca mulatta			adult	(Agarwal et al., 1964b)
HOMINIDAE (PRIMATES)				
Homo sapiens			adult	(Agarwal et al., 1964b)

iris margin (Stone, 1967). The lens is regenerated by the iris epithelium (Colucci, 1891; Yamada et al., 1973; Yamada and McDevitt, 1974), which is of neuroectodermal origin. Therefore, in this case the developmental field and the regeneration field do not correspond. Another regeneration pathway which does not correspond to the developmental pathway is lentoid formation from the pigment retina, which requires special experimental conditions, such as repeated wounding (Fischel, 1900) of the pigment retina, removal of the iris after lentectomy (Fischel, 1900, 1903), insertion of objects into the pigment retina (Sato, 1951; Stone, 1960), or cell culture conditions (Eguchi, 1979).

Lens regeneration potency is maximal at the dorsal pupillary margin of the iris epithelium in the newt, and decreases laterally (Sato, 1930; Mikami, 1937, 1941b; Stone, 1954) as well as posteriorly towards the ciliary epithelium (Okada, 1935). However, even the mid-ventral iris epithelium is capable of lens regeneration if separated from the dorsal iris by a pliofilm (Stone, 1955), or a celluloid membrane (Ciaccio, 1933), or after treatment with ultraviolet irradiation (Dürken-Woitzik, 1950), or the carcinogen N-methyl-N'-nito-N-nitroso-guanidine (Eguchi and Watanabe, 1973). Multiple lenses can be obtained if celluloid septae are inserted radially through the iris (Ciaccio, 1933). Furthermore, ventral iris epithelial cells form lentoid bodies under conditions of cell culture (Eguchi, Abe and Watanabe, 1974). It seems that there are at least two different populations of pigmented iris epithelial cells in the newt, which can only be distinguished according to their lens regeneration potency.

The faster regenerating cells are concentrated at the dorsal iris margin (Watanabe, 1978), and seem to have a higher mitotic rate after lentectomy (Yamada et al., 1975).

4. CELL TYPE CONVERSION DURING LENS REGENERATION FROM THE IRIS

Lens regeneration from iris epithelial cells (Colucci, 1891; Yamada et al., 1973), and neural retina regeneration from pigment retina cells (Colucci, 1891; Keefe, 1973a,b,c) are unambiguous examples of cell type conversion, also called cellular metaplasia, in adult vertebrates. During embryonic development cells become more and more specialized or differentiated, and parallel to this process determination or loss of the original totipotency or multipotency of cells occurs. Upon cell division, most adult vertebrate cells can only give rise to cells of their own kind. For instance, lens epithelial cells give rise to other lens cells, and pigment retinal cells to pigment retinal cells. Terminal differentiation is only reached by few cell types, such as nerve cells and lens fiber cells, which do not normally divide any more (Papaconstantinou, 1967). However, even terminally differentiated cells may regenerate parts of their cell body. For example, nerve cells of the neural retina can regenerate their axons in the optic nerve (Turner and Singer, 1974), and photoreceptor cells their rod outer segments (Young, 1967). Lens fiber cells, however, loose their nuclei, and their physiological abilities become very limited.

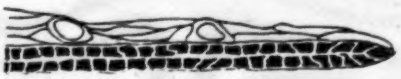
The term transdetermination (Hadorn, 1966) has been used to describe cell type conversion under experimental conditions into cell types outside their cell lineage. A similar term transdifferentiation has been applied to the conversion of retinal pigmented epithelial cells into lens cells in cell culture (Eguchi, 1979).

Programmed cell death occurs during the detachment of the newly regenerated lens from the dorsal iris epithelium (Yamada, 1977). This can be regarded as an extreme case of cell differentiation.

5. MORPHOLOGICAL DESCRIPTION OF THE LENS REGENERATION STAGES IN THE ADULT RED SPOTTED NEWT

In different developmental stages of embryonic to adult newts (Monroy, 1937) of different species the size (Monroy, 1937), weight, and cell number (Sato, 1940) of lenses vary. If the lens is removed, the speed of its regeneration is also affected by the temperature (Nakamura, 1935). Therefore, a series of independent morphological criteria are necessary to assess the degree of lens differentiation. Sato (1940) established 13 morphological lens regeneration stages for the fire-belly newt (*Cynops pyrrhogaster*), adults and larvae, and the larvae of the common newt (*Triturus vulgaris*). These stages have been adapted for the larvae of the crested newt (*Triturus cristatus*) by Zalokar (1944a), for the larvae of the red spotted newt (*Notophthalmus viridescens*) by Reyer (1948, 1954), and for the adult animals of this species by Yamada (1967). The staging system of Yamada (1967) has been used because the same species and groups of similar age span have been investigated in this thesis. A description of these regeneration stages (Figure 3) is given below, with a few minor modifications. The stages 12 and 13 of Sato (1940) have been omitted by Yamada (1967), but they are nevertheless described here. Also, the normal iris is treated as stage 0. The time course of lens regeneration is indicated in days

Figure 3. Morphological lens regeneration stages in the adult newt *Notophthalmus viridescens* according to Yamada (1967), with the addition of stages 12 and 13 according to Reyer (1954) and Sato (1940), and the normal iris, here called stage 0.



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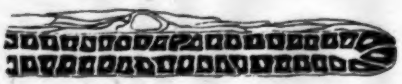

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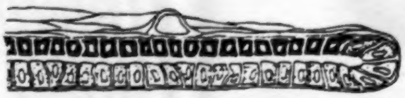

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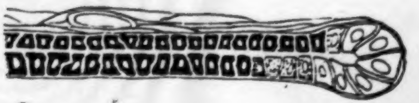

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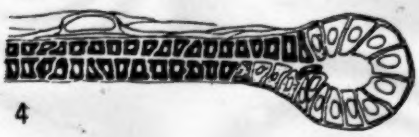

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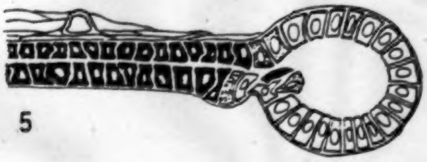

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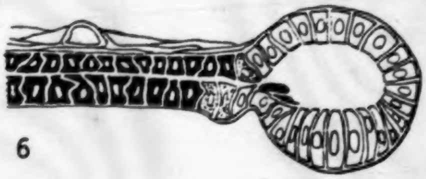

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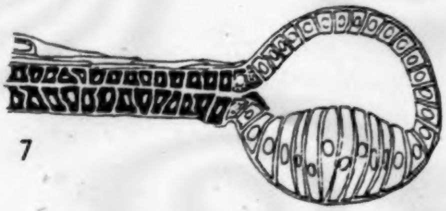

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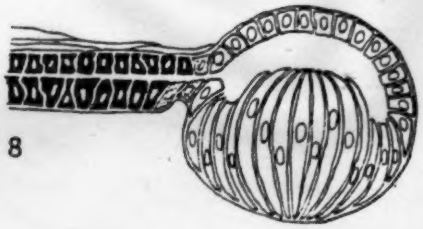

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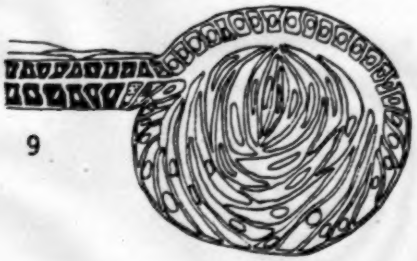

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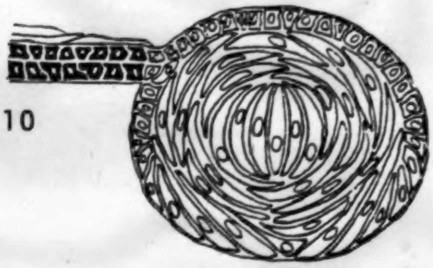

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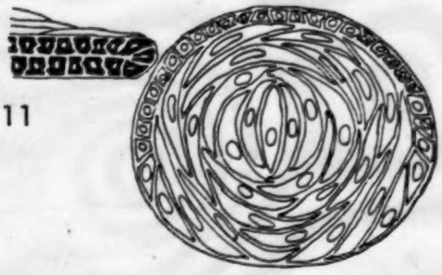

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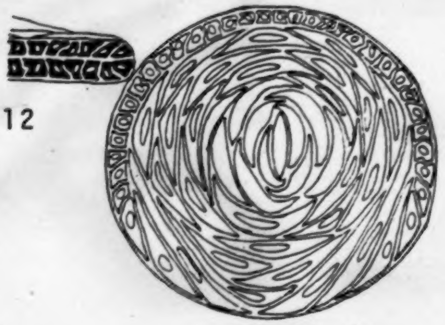

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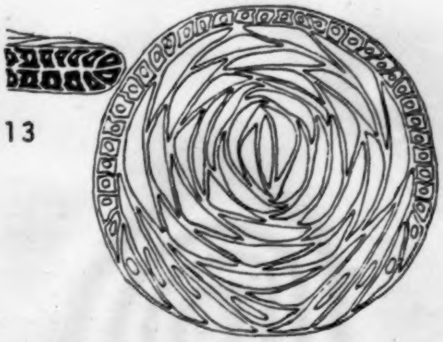


13

after lentectomy for a temperature of 21 to 22°C.

Stage 0 (before lentectomy). The totally pigmented dorsal iris epithelium is thin, and the cells are small. The interlaminar space is not greater than the intercellular spaces within the lamina. The nuclei are small, with many invaginations, and they are not visible in 7 μm sections.

Stage 1 (day 3 to 6). The interlaminar space is slightly enlarged. The invaginations of the nuclear envelopes are less prominent due to nuclear swelling in iris epithelial cells. A few macrophages start to invade the iris epithelium.

Stage 2 (day 6 to 10). The melanosomes or pigment granules of dorsal iris epithelial cells are reduced, often throughout the inner lamina, leaving the cells largely depigmented. The number of macrophages is increased.

Stage 3 (day 8 to 11). A group of almost completely depigmented enlarged cells forms at the dorsal iris margin. Large portions of the internal lamina have repigmented.

Stage 4 (day 9 to 15). The depigmented group of cells at the dorsal iris margin forms a sickle-shaped lens vesicle along the dorsal iris margin (Connelly, 1977d, 1978), which is connected to the external and internal laminae of the iris epithelium. The cavity of the lens vesicle is continuous with the interlaminar space of the iris. In *N. viridescens* often a small number of pigmented degenerating cells from the internal lamina can be observed in the vesicular cavity.

Stage 5 (day 12 to 15). The lens vesicle is more spherical and enlarged by addition of depigmented cells from the internal lamina of the iris, as well as by proliferation of cells within the lens vesicle. The retinal side of the lens vesicle is slightly thickened. There is often a slight groove where the lens vesicle joins the internal lamina of the iris epithelium.

Stage 6 (day 12 to 16). On the retinal side of the lens vesicle the primary lens fiber cells start elongating and become acidophilic. Mitotic activity ceases in elongating cells, but is high in all other cells of the lens vesicle which exhibit a basophilic staining reaction. Depigmented cells still enter the lens vesicle from the internal lamina of the iris epithelium.

Stage 7 (day 15 to 18). The internal face of the inner layer of the lens vesicle protrudes into the lens vesicle cavity, forming a primary lens fiber hillock.

Stage 8 (day 15 to 19). The lens fiber core has grown at the expense of the lens vesicle cavity, which becomes reduced to a narrow slit. In the equatorial region, between the elongated primary fiber cells and the cuboidal lens epithelial cells, the first generation of secondary lens fibers starts to elongate.

Stage 9 (day 18 to 20). The lens vesicular cavity has almost vanished, and the lens fiber core has grown by cell elongation and addition of new secondary lens fiber cells. The cells on the corneal side become now flattened to form the lens epithelium, and start to surround the lens fiber core. Mitotic figures are frequent in the lens epithelium and lens stalk, which still connects the lens to the iris. In the primary lens fiber cells, the nuclei start to degenerate.

Stage 10 (day 18 to 25). The lens epithelium lies firmly on the enlarged lens fiber core, and is still connected to the dorsal iris. In the primary lens fiber cells, the nuclei start to degenerate.

Stage 11 (day 21 to 28). The lens is disconnected from the dorsal iris, and the cells of the lens stalk have died. The nuclei in the primary lens fibers have become as acidophilic as the surrounding cytoplasm and are compressed.

Stage 12 (day 25-35). The nuclei in the primary lens fiber cells have disappeared. The lens has become bigger in size.

Stage 13 (day 30 to several months). The nuclei in the secondary lens fibers have also disappeared, except in a few young secondary fibers in the outermost portion of the lens.

These lens regeneration stages have been grouped as follows (Sato, 1940; Zalokar, 1944a; Reyer, 1954):

I latent period = stages 1 and 2
II initial period, formation of the lens vesicle = stages 3 to 6
III period of lens fiber differentiation = stages 7 to 11
IV period of growth = stages 12 and 13

Stages 1 and 2 of the latent period are not confined to the dorsal iris margin, where the lens forms. Cells of the ventral iris epithelium repigment after they have reached stage 2. Therefore, we have called these stages nonspecific lens regeneration stages (Cuny and Zalik, 1981).

The morphological staging system is applicable to lens regenerates which have been obtained from irises in organ culture (Connelly et al., 1973; Yamada et al., 1973; Cuny and Zalik, 1981).

6. SOME CELLULAR EVENTS DURING LENS REGENERATION *IN VIVO*.

ACCUMULATION OF CELL TYPE SPECIFIC MOLECULES

Removal of the lens through a horizontal incision in the cornea of the adult newt leads to sudden breakdown of the intraocular pressure (Reinke, 1906; Wachs, 1914). This immediately causes the dilation and possibly fenestration of the capillaries in the iris stroma or changes their permeability (Cole, 1977; Raviola, 1977). Within three to six days in *Notophthalmus* (Yamada, 1967) or about one day in *Cynops* (Eguchi, 1963) iris epithelial cells swell. This is stage 1 of lens regeneration, the thickening of the iris epithelium. Although the iris epithelium is not wounded during the operation (Wolff, 1894, 1895), blood serum and blood cells enter the eye chambers and an inflammatory reaction occurs (Fischel, 1900; Eguchi, 1963). Leukocytes (Wolff, 1894, 1895; Fischel, 1897), especially polymorphonuclear leukocytes (Eguchi, 1963), platelets, erythrocytes, granulocytes, lymphocytes, monocytes, mast cells from the limbus and uvea (Eguchi, 1963), and in particular histiocytes or macrophages (Eguchi, 1963; Dumont and Yamada, 1972) begin surrounding and penetrating the iris epithelium, especially at its inner dorsal surface (Wolff, 1895; Eguchi, 1963). In the intact eye, dorsal iris epithelial cells have more mitochondria than the ventral iris epithelial cells (Eguchi, 1963). In dorsal and ventral iris cells the cell surface charge density, measured by cell electrophoresis, increases at 1 to 3 days after lens removal (Zalik and Scott, 1972), but after 10 to 15 days this charge decreases and becomes slightly lower than in cells of unoperated eyes. This decrease can partially be attributed to loss of sialic acid containing molecules at the cell periphery (Zalik and Scott, 1973).

The nuclei of iris epithelial cells in the intact eye are small and wrinkled, and have many lobes and invaginations (Eguchi, 1963). They start to swell three days after lens removal at stage 2 (Eguchi, 1963; Ogawa, 1962), and blebs start to form on the nuclear membrane (Karasaki, 1964). The first sign of nuclear change, however, is the enlargement of the nucleolus that occurs two days after lentectomy. The nucleolus increases in size up to day 4 after lens removal (Dumont et al., 1970). This is one of the manifestations of an increased transcription of ribosomal ribonucleic acid. Indeed, synthesis of a precursor of 3.1 x 10^6 daltons for 18S (0.7 x 10^6 daltons) and 28S (1.4 x 10^6daltons) ribosomal ribonucleic acid (Reese, 1977) as well as 4S ribonucleic acid become detectable by ^{14}C-uridine incorporation at this time (Reese et al., 1969). This synthesis is accompanied by an increased uptake of inorganic phosphate, measured with radioactive $^{32}PO_4$, by iris epithelial cells. This uptake is higher in the dorsal part of the iris than in the ventral part (Eguchi and Ishikawa, 1960). It correlates in time with an elevated alkaline phosphomonoesterase activity (Eguchi and Ishikawa, 1960). These results suggest that the phosphate metabolism is increased. Ribosomal ribonucleic acid synthesis is enhanced by both increasing the rate of transcription as well as by ribosomal ribonucleic acid cistron amplification (Collins, 1972). After depigmentation at stages 2 to 3, free ribosomes as well as rough and smooth endoplasmic reticulum become much more prominent in iris epithelial cells (Eguchi, 1963); polyribosomes have been detected at stages 5 to 6 of regeneration (Eguchi, 1963).

In the intact eye, iris epithelial cells do not divide (Yamada and Roesel, 1969). Synthesis of deoxyribonucleic acid starts 4 days after lentectomy at stage 1 (Eisenberg and Yamada, 1966), when a few mitotic figures become detectable (Yamada and Roesel, 1971). The duration of the cell cycle has been studied by several investigators, and has been reported to be in the range of 23 (Mitashov, 1969b), 45 (Michel and Yamada, 1974) to 67 hours (Eisenberg Zalik and Yamada, 1967) *in vivo*. There are two major peak periods of ^3H-thymidine incorporation at 7 and 12 days after lentectomy (Eguchi and Shingai, 1971), indicative of synthesis of deoxyribonucleic acid; they correlate with two peaks of mitotic division frequency (Yamada and Roesel, 1971; Yamada, 1977), the first one occurring around 7 days after lentectomy (stages 2 to 3) during lens vesicle formation, and the second one at around 12 to 15 days (stages 4 to 6) at the beginning of lens fiber differentiation. The mitotic activity decreases at 20 days of lens regeneration (Yamada, 1977).

Protein synthesis is enhanced starting at day 3 of lens regeneration and increases in two peak periods (Yamada and Takata, 1963) which follow the previously mentioned mitotic peaks. The kinds of proteins synthesized seem to shift from iris specific to lens specific proteins. However, some proteins maintain the same level of concentration throughout lens regeneration; tyrosine aminotransferase, creatinine phosphokinase, and isocitrate dehydrogenase do not change their activities (Yamada, 1977). The activity of tyrosinase is low in iris epithelial cells; it increases about 4 to 6 fold during lens regeneration to reach a peak in young lens regenerates of about 100 fold the activity of the normal iris. This enzyme then decreases its activity in young lenses to about twice that of the iris epithelium at 150 days after lens removal (Achazi and Yamada, 1972; Yamada, 1977). Galactosyl transferase is another example of an enzyme which has a very high activity (47 pmoles galactose ^{14}C/mg DNA/hr) in young fully regenerated lenses; its activity is increased 11 fold as compared to the activity of the normal iris (3 pmoles galactose ^{14}C/mg DNA/hr)(Idoyaga-Vargas, Yamada and Michel, 1976). In the normal iris the activities of alkaline phosphomonoesterase (Pósalaky et al., 1951) and glucosaminidase (Idoyaga-Vargas and Yamada, 1974) are low. The activity of alkaline phosphomonoesterase reaches a peak at 14 days of lens regeneration (Pósalaky et al., 1951), and the activity of glucosaminidase after 17 days. Glucosaminidase activity is higher in the dorsal iris than in the ventral iris,and also higher than in the ocular fluids (Idoyaga-Vargas and Yamada, 1974). The synthesis of an iris specific protein has not yet been studied in detail, but melanin synthesis is probably minimal during lens regeneration. Iris epithelial cells of the internal lamina also undergo depigmentation. Cells actively extrude their melanosomes during the initial inflammatory response (Wolff, 1895; Fischel, 1900; Eguchi, 1963; Dumont and Yamada, 1972). These extruded organelles are engulfed by macrophages by phagocytosis either directly from the iris cells (Eguchi, 1963), or from the intercellular space (Fischel, 1900; Dumont and Yamada, 1972). Beside exocytosis or extrusion of cytoplasm of iris epithelial cells autophagy of specific cytoplasmic components also occurs. This process is detectable as early as regeneration stages 5 to 6 (Eguchi, 1963), and is an important mechanism of dedifferentiation (Yamada et al., 1978). No detectable lens specific proteins are synthesized in the dorsal iris epithelial cells during the first wave of protein synthesis which occurs after lens removal (Titova, 1957; Ogawa, 1962; Takata et al., 1964a,b), and persists up to the onset of lens vesicle formation. However, the second wave of protein synthesis occurring during the formation and elongation of the lens fibers leads to accumulation of the lens specific proteins, the crystallins (Kobayashi, 1926; Titova, 1957; Ogawa, 1962; Takata et al., 1964a,b). As this process occurs in the cytosol, the lens fiber cells become filled with a fibrous matrix (Eguchi, 1963).

7. LENS SPECIFIC PROTEINS, THE CRYSTALLINS

Four gene families of crystallins are known. Two families, the α- and β-crystallins are present in all vertebrates (Clayton, 1970). One family of these proteins, the δ-crystallins are only found in birds and "reptiles" (Clayton, 1979; Yu et al., 1977), while γ-crystallins are found in the remaining vertebrates (Clayton, 1970; 1979). In precattle γ-crystallins emerge first during lens development, and predominate in the embryonic lens; after birth and in the adult lenses α-crystallins predominate (Clayton, 1974); 75% of the lens protein production is due to the synthesis of α-crystallins (Van der Ouderaa et al., 1974). In the embryonic lens of the leopard frog (*Rana pipiens*) lens proteins of the non-γ-crystallin type, presumably β-crystallins (Clayton, 1970), emerge before the γ-crystallins (McDevitt, 1967); the latter are first restricted to lens fiber cells (Brahma and McDevitt, 1974). During lens regeneration in the newt, β-crystallins are also synthesized before or simultaneously with the γ-crystallins (Takata et al., 1965, 1966; Yamada, 1967; McDevitt and Brahma, 1979, 1981; McDevitt and Yamada, 1980). The concentrations of subfamilies of crystallins vary independently during development (Papaconstantinou, 1967; Clayton, 1970). In *Xenopus laevis* for instance, one band of α-crystallin, 5 of β-crystallins, and 7 of γ-crystallins have been resolved by thin layer isoelectric focussing of soluble lens extracts from larval and adult individuals (Brahma and Bours, 1972). Densitometric tracings of the gel bands revealed that the concentration of α-crystallin is low during larval life (0.5%) and increases in the adult lens to 7.3% of the total crystallin concentration. Beta-crystallin is the major lens protein in the adult lens, contributing 52.1% to the total crystallins. This protein is not as abundant as γ-crystallin in the larval stage when it only accounts for 23 to 37.8% of the lens proteins. Gamma-crystallin is the major protein of the larval lens making up 76.5% of the total crystallin content; it decreases to 40.6% in the adult lens (Brahma and Bours, 1972). Decrease in γ-crystallin content during postembryonic development was also reported from the rat (Lerman et al., 1966). In *Rana catesbeiana* after metamorphosis, the γ-crystallin concentration of the lens also decreases. In contrast to this, α- and β-crystallin concentrations increase, and the electrophoretic mobility of α-crystallin increases, suggesting that the activity of different α-crystallin genes may be changed (Polansky and Bennett, 1970, 1973). In the two year old adult *Rana clamitans*, however, 78% of the lens crystallins are still of the γ-crystallin type (Clayton, 1974). The β-crystallins are the most heterogeneous group in cattle and human lenses (Bours, 1971). The primary structure of all crystallin families has been well preserved during evolution, so that antibodies prepared against *Xenopus laevis* crystallins can cross-react with crystallins of *Bufo bufo, Rana esculenta, Triturus cristatus* and *Ambystoma mexicanum*. However, *X. laevis, T. cristatus* and *A. mexicanum* crystallins produce spurs upon immunoelectrophoresis with anti-*R. esculenta* lens antiserum, indicating only partial cross-reactivity (Brahma and Van Doorenmaalen, 1969). Nevertheless, anti-*Rana pipiens* γ-crystallin antibodies can react specifically with γ-

Rana pipiens have predominantly γ-crystallins, especially in the fiber core (McDevitt, 1967; McDevitt et al., 1969). The same may be true for newly regenerated lenses of *Notophthalmus viridescens* (Cuny and Zalik, 1981; Takata et al., 1965, 1966). Beta-crystallins seem to increase during aging in most vertebrates, including birds (Clayton, 1970), and take over the space previously occupied by γ- or α-crystallins.

The chemistry of bovine (Bloemendal, 1977) and chicken crystallins (Clayton, 1969, 1979) has been investigated in remarkable detail, but the amphibian crystallins need further attention (Clayton, 1974; Brahma and McDevitt, 1972b, 1974). Alpha, β-, and γ-crystallins occur in soluble form in the cytosol, but in the bovine lens and most likely in all other vertebrate lenses, α-, β-, and γ-crystallins can also form insoluble crystallites (Malinowski and Manski, 1980) via molecular alignment mediated by phosphopeptides of low molecular weight (Bettelheim and Wang, 1977). Bovine and rat crystallins have been reported to be glycosylated in the degenerating lens fibers of cataractous lenses. This may be related to opacification of the lens (Stevens et al., 1978).

Alpha-crystallins occur as heteropolymers of two subunit gene product subfamilies αA and αB (Clayton, 1979), with 57% homology in their amino acid sequences (Van der Ouderaa et al., 1974). In these sequences seven not correlating gaps occur between the homologous portions (Van der Ouderaa et al., 1973). In cell-free systems αA- and αB-subunits assemble automatically; the αA-crystallin subunit appears to self-assemble, and the αB-subunit, if present, is incorporated into the resulting high molecular weight HMα-complex (Asselbergs et al., 1978). The insoluble HMα-crystallin, the major crystallin in the calf lens (Clayton, 1979), ranges in molecular weight from 1×10^5 (Bloemendal, 1977), or 30.4×10^5 (Chiou et al., 1979) to several million daltons (Bloemendal, 1977). The soluble α-crystallin has a molecular weight of 5.83×10^5 daltons (Chiou et al., 1979). A common bicistronic messenger ribonucleic acid is involved in the translation of the αA2- and αB2-subunits; the 14S cistron codes for the αA2-subunit and the 10S cistron for the αB2-subunit (Asselbergs et al., 1978). The precursor of the two subunits contains 50 additional amino acid residues, and is cleaved posttranslationally (Chen and Spector, 1977). The αA1- and αB1-subunits are derived from the αA2- and αB2-chains by posttranslational deamination, a process not yet found in the embryo (Bloemendal, 1977). In bovine lenses the αA2-crystallin subunit contains 173 amino acid residues and has a molecular weight of 19830 daltons (Van der Ouderaa et al., 1973); the αB2-subunit has 175 amino acids and a molecular weight of 20070 daltons (Van der Ouderaa et al., 1974). The α-crystallin family is the most anodal of the lens proteins with isoelectric points ranging between pH 4.5 to

5.1 (Bours, 1971; Clayton, 1974). In chickens, for example, the isoelectric points of α-crystallins vary from 4.52 to 4.87 (Brahma and Van der Starre, 1976). Upon excitation with ultraviolet radiation, α-crystallin exhibits blue fluorescence with a maximum fluorescence at a wavelength of 420 nm (Fujimori, 1978). Alpha-crystallin-like molecules of 2.6×10^4 and 3.4×10^4 daltons have been noticed in lens cell membranes (Boemendal, 1977).

The β-crystallins are also heteropolymers of two subunit gene product families (Clayton and Truman, 1974; Clayton, 1974, 1979) as judged by the antigenic properties of the six major subunits of this protein found in the chicken lens (Clayton, 1979). Calf β-crystallins do not self-assemble (Asselbergs et al., 1979), and monomeric β-crystallin has been reported in chickens after hatching (Brahma and Van der Starre, 1976; Clayton, 1979). This monomeric form in the chicken is similar to the most cathodal member of the bovine β-crystallin subfamily, the β_s-crystallins, which do not seem to have an N-terminal amino acid (Kabasawa et al., 1977). The β_s-crystallins have isoelectric points between pH 6.70 and 6.90 (Bours, 1971), and a molecular weight of about 2.8×10^4 daltons (Kabasawa et al., 1977). The β_s-crystallins can assemble to βL-crystallin dimers of 45.9×10^3 daltons molecular weight (Chiou et al., 1979). The high molecular weight bovine βH-crystallin has a molecular weight of 15.8×10^4 daltons (Chiou et al., 1979). The βH-crystallin has isoelectric points ranging between pH 5.69 and 6.60 in bovine (Bours, 1971) or 7.03 in chicken lenses (Brahma and Van der Starre, 1976).

A single gene family codes for γ-crystallins (Clayton, 1979).

Gamma-crystallins are globular monomers (Carlisle et al., 1977) when in solution. They form the major insoluble crystallin fraction in the lens of the adult rat (Clayton, 1974). They are the smallest crystallins with reported average molecular weights of 13.5×10^3 daltons in the mackerel (Clayton, 1974), 20×10^3 (Kabasawa et al., 1977) to 21.4×10^3 daltons in calves and cows (Chiou et al., 1979), or 24×10^3 daltons in adult cattle (Kabasawa et al., 1977). The γ-crystallins are the most cathodal crystallins with isoelectric points between pH 7.00 and 8.10 in cattle (Bours, 1971), pH 7.8 and 8.0 in *Rana pipiens, Rana temporaria* and *Ambystoma mexicanum,* and pH 7.15 to 8.30 in *Xenopus laevis* (Brahma and Bours, 1972). When examined electrophoretically the γ-crystallins separate into 4 components in *Rana catesbeiana* (Polansky and Bennett, 1970, 1973), *R. pipiens* (McDevitt, 1967) and *R. temporaria* (Brahma and McDevitt, 1974), into 7 bands in *Xenopus laevis* (Brahma and Bours, 1972), while in the newt *Notophthalmus viridescens* these crystallins are only represented by one band (Nöthiger et al., 1971; Clayton, 1974). In bovine lenses 4 antigenically similar γ-crystallin fractions called γI, γII, γIII and γIV (Björk, 1964), have been distinguished (Papaconstantinou, 1967; Brahma and McDevitt, 1974). All of these polypeptides carry an N-terminal glycine (Björk, 1964; Kabasawa et al., 1977), and are characterized by a high cysteine content (Slingsby and Croft, 1978). Microheterogeneity was found in all of these γ-crystallin fractions, except for the homogeneous fraction γII (Björk, 1964). The fractions I and IVa have not yet been purified to homogeneity, but fractions IIIa, IIIb and IVb are electrophoretically pure (Björk, 1964), and can form tetragonal crystals in cold phosphate buffer (Björk, 1964). The molecular weights of the pure γ-crystallin fractions have been determined as 19.1 x 10^3 daltons for γII, 20.9 x 10^3 daltons for γIIIa, 19.9 x 10^3 daltons for γIIIb, and 20.8 x 10^3 daltons for γIVb (Björk, 1964). There is no α-helical coiling in the secondary structure of bovine γ-crystallin (Horwitz et al., 1977). The γ-crystallin fraction II is easy to purify, and it is better known than the other fractions. The γ-crystallin fraction II is a polypeptide of 165 amino acid residues, and the molecular weight calculated from the amino acid sequence is 19.8 x 10^3 daltons (Carlisle et al., 1977). When the tetragonal crystal of fraction II is examined by X-ray diffraction two globular domains of 25 Å diameter are detected within the γII molecule (Blundell et al., 1978). Portions of the amino acid sequences of the γ-crystallin fractions IVa and IVb are also known (Slingsby and Croft, 1978). Two sites of microheterogeneity among the fraction IV molecules have been found close to the C-terminal ends. This may indicate that two nonallelic cistrons code for γ-crystallin IVa and IVb (Slingsby and Croft, 1978).

Delta-crystallins are not present in the newt, but may be briefly introduced here. They are tetramers (Piatigorsky et al., 1974) and are products of at least two genes (Bhat et al., 1980); they are not related antigenically to other crystallins. Delta-crystallins are hard to dissociate into their subunits (Clayton, 1979). There are three major fractions of aggregated δ-crystallins in the hatched chicken (Clayton, 1979), but only two in the chick embryo (Reszelbach et al., 1977). Their secondary structure is mainly α-helical (Yu et al., 1977). Their molecular weights range from 15.5 x 10^4 to 16.5 x 10^4 daltons

(Clayton, 1970); they have also been reported as higher molecular weight aggregates of 46 x 10^4 daltons (Clayton, 1970). The molecular weight of all subunits is initially 50 x 10^3 daltons in the chick embryo. These subunits are convertible into two acidic tryptic peptides. One of them, the 48 x 10^3 dalton fragment, is identical with the post-translational modification of the 50 x 10^3 dalton subunit (Shinohara et al., 1980). The molecular weight of subunits in the adult chicken is reduced to 43 x 10^3 daltons (Alcalá et al., 1977). The isoelectric points of δ-crystallins range from either pH 5.2 to 5.36 (Bours, 1971), or 5.12 to 5.33 (Brahma and Van der Starre, 1976). Delta-crystallins can occur as integral proteins in cell membranes of chick lens fibers (Alcalá et al., 1977).

8. SUGGESTIONS FOR THE CONTROL MECHANISM REGULATING LENS REGENERATION FROM THE DORSAL IRIS

The following sections review some of the ideas about how lens regeneration from the dorsal iris can be stimulated after lens removal.

8.1 ENVIRONMENTAL AND SYSTEMIC INFLUENCES

Since lens regeneration consistently occurs at the dorsal iris margin in the newt, Wolff (1895) and Fischel (1900) thought that gravity may exert some control over this process. However, Wolff (1901) managed to keep a lentectomized newt lying on its back for about a month by cutting its spinal cord, and lens regeneration still occurred from the dorsal iris margin. Wachs (1920) and Sato (1933) rotated the eye balls in the orbits of newts 180° without cutting the optic nerve; they still obtained lens regeneration from the dorsal iris margin now located in ventral position. Thus, gravity does not influence lens regeneration. Brachet and Benoit (1899), Fischel (1897, 1900), and Rübsaamen (1950) considered that the iris was part of a light receptor apparatus, and wondered whether light conditions may influence lens regeneration. Newts kept in constant dark or constant light regenerated lenses equally well (Rübsaamen, 1950; Nikitenko, 1950; Chun and McCarthy, 1980). When newt larvae of the species *Triturus vulgaris* and *Pleurodeles waltli* were kept under conditions of dim light or of total darkness, regeneration was retarded and degeneration of lens fibers was evident in normal lenses as well as those regenerating after lentectomy (Borsuk and Popov, 1961, 1968). In spite of the latter results, it seems that light is not involved directly in the control of lens regeneration.

Newts can regenerate their lens throughout the year. Nakamura (1935) investigated the influence of the season and temperature on lens regeneration in *Cynops pyrrhogaster*. In April at an average temperature of 12.5^{0}C, depigmentation started 30 days after lens removal, but in July at 27.5^{0}C this process started 10 days after lentectomy and lens fibers were present after 15 days. In July, when newts were kept in a refrigerator at 5 to 8^{0}C, depigmentation did not start before day 30, and in April when newts were kept in an incubator at 27.8^{0}C lenses with lens fibers were present at 15 days after lentectomy. Nakamura (1935) concluded that the temperature was the major environmental factor affecting the speed of lens regeneration, and that the time of the year was unimportant. Mikami (1941a,b) and Donaldson and Chan (1973) essentially confirmed Nakamura's conclusion, although a slightly faster regeneration rate was noticed in summer time. Collins (1974b) reports an increase in affinity for nitrocellulose of the deoxyribonucleic acid of dorsal iris epithelial cells during summertime. Only lens regeneration stage 5 was reached 60 days after lentectomy if newts (*Notophthalmus viridescens*) were kept at a constant temperature of 12°C. This contrasts with lens regeneration stage 11 obtained after 50 days when animals were kept at 18°C. Although a temperature of 34°C was lethal in 73% of the newts, in the surviving animals lenses regenerated as fast as in newts kept at 26°C; they reached stage 11 at 20 days after lentectomy (Donaldson and Chan, 1973). Lenses still regenerate after repeated removal (Ciaccio, 1934; Nikitenko, 1939), and the speed of lens regeneration is not accelerated or slowed down by slightly earlier lentectomy in the contralateral eye (Nikitenko, 1951a). Eyes grafted into the belly region during the embryonic state and with no connections to the brain will regenerate a lens after lentectomy later in life (Mangold, 1931). Lens regeneration is faster in premetamorphic larvae and slows down during metamorphosis and in postmetamorphic individuals when compared to the normal larvae or the adults of *Triturus vulgaris* and *T. alpestris* (Monroy, 1937; Wachs 1920a) *Notophthalmus viridescens* (Reyer, 1948, 1954a) or *Cynops pyrrhogaster* (Sato, 1940).

8.2 CONTROL OF THE CELL CYCLE

If mitotic division is prevented by X-ray or ultraviolet irradiation, the iris can no longer regenerate a lens (Dürken-

Woitzik, 1950; Borsuk, 1957; Donaldson, 1972), although the invasion of macrophages into the iris continues under these conditions (Michel and Yamada, 1974). If a previously irradiated iris incapable of mitotic division is grafted into an unirradiated eye, lens regeneration does not occur; an unirradiated iris, however, can regenerate a lens when implanted into an irradiated eye (Donaldson, 1972). Thus, mitotic division in the iris is essential for lens regeneration, although it is not necessary in the tissues which are assumed to stimulate lens regeneration. Yamada et al. (1975), and Yamada and Beauchamp (1978) have suggested that the basic requirement for the conversion of iris epithelial cells into lens cells is their passage through a definite number of mitotic cell cycles. Agents which could accelerate the cell cycle would enhance the chance of successful lens regeneration.

8.3 WOUNDING STIMULUS

Earlier investigators in this field believed that lens regeneration could only occur after wounding of the iris (Colucci, 1891; Fischel, 1900, 1903; Weber, 1918). However, experiments (Wolff, 1894, 1895, 1903) showed that lens regeneration could be obtained from intact irises after careful removal of the primary lens through a horizontal incision in the cornea or through the retina and sclera. Wolff (1903) and Stone (1943, 1952) cut the iris without removing the lens, and no lens regeneration occurred. In fact, if the iris is wounded in the lentectomized eye, irregular or supplementary lenses are produced (Stone, 1954; Stone and

Griffith, 1954), and lens regeneration is slower (Ikeda and Amatatu, 1941). Reinke (1906) suggested that although no direct mechanical wounding of the iris occurred during Wolff's procedure, the intraocular pressure was nevertheless abruptly lowered when the eye was opened. Wolff (1895) himself noticed the inflammatory response of the iris later reported by Eguchi (1963) when this worker investigated the invasion of leukocytes into the iris tissue. In the rabbit eye inflammatory responses to wounding are partially mediated by prostaglandin E_2 (Cole and Unger, 1973; Raviola, 1977) or E_1 (Pedersen, 1980). However, Connelly (1977a) could not suppress lens regeneration by intraperitoneal injection of indomethacin, a well known inhibitor of prostaglandin synthesis. Finally, Politzer (1937) destroyed the lens in the intact newt eye using X-rays, without opening the eye, and lens regeneration occurred normally. The evidence presented above seems to indicate that the wounding stimulus is not sufficient for lens regeneration.

8.4 INHIBITION OF LENS REGENERATION VIA LENS CONTACT

As soon as the lens is missing, the iris will regenerate a new one. Fischel (1900) reasoned that direct contact of the iris epithelium with the lens capsule inhibited lens regeneration in the intact eye. He implanted bread or boiled potato balls to fill the pupillary space of a lentectomized eye after lens removal, but his data were too variable to be conclusive. Wachs (1914) and Kesselyak (1936) in a similar experiment implanted the more inert paraffin balls, but could not prevent lens regeneration in most cases. Similarly, Mikami (1941b) implanted newt liver, heart, brain or neural retina into lentectomized eyes. He obtained the best degree of inhibition of lens regeneration with heart tissue, possibly because of its most fibrous consistency. One of the most popular experiments in lens regeneration research has been the reimplantation of living newt lenses into the lentectomized eyes (Wachs, 1914; Kawakami, 1941; Ikeda and Amatatu, 1941; Mikami, 1941b; Dinnean, 1942a,b; Zalokar, 1942, 1944a; Ikeda and Kojima, 1940; Uno, 1943; Stone, 1943; Takano et al., 1958; Reyer, 1959, 1966; Frost, 1961; Ogawa, 1964). Despite these numerous attempts, the results remained inconclusive. It seems that if these lenses are not yet fully regenerated or too small in size they cannot suppress lens regeneration completely (Wachs, 1914; Frost, 1961). In addition, larger lenses cannot prevent lens regeneration when they are not properly placed in the pupil (Wachs, 1914). On the other hand, cataractous lenses with damaged lens fibers (Mikami, 1941b; Dinnean, 1942a,b), or implanted lenses which do not touch the iris (Zalokar, 1944a) can in some instances completely suppress lens regeneration. Spirito and Ciaccio (1931), Takano et al. (1958), and Eguchi (1961) have succeeded in displacing the lens from the pupil posteriorly into the eye by inserting a glass or tungsten rod between the dorsal iris and lens through the sclerocorneal junction; small secondary lenses regenerated in the presence of the primary lens. Stone (1957) also reported one case of an accidentally dislodged lens, where a second lens had regenerated.

8.5 SECRETION EQUILIBRIUM OF A STIMULATOR FROM THE RETINA AND AN INHIBITOR FROM THE LENS

In analogy to embryonic lens induction, Spemann (1905) proposed a hypothesis for lens regeneration in the newt. He suggested that the neural retina constantly secretes a lens regeneration stimulating substance which diffuses through the eye chambers to reach the dorsal iris. The lens, on the other hand, produces a lens regeneration inhibiting substance, counterbalancing the retinal influence. As soon as the lens is removed, the equilibrium of these antagonizing factors is disturbed and lens regeneration is stimulated by the retina. Removal of the neural retina along with the lens delayed lens regeneration by 5 to 8 days. It only continued (Stone and Steinitz, 1953a) when the new neural retina had partially regenerated (Wachs, 1920a; Zalokar, 1944a; Hasegawa, 1958, 1965; Stone and Steinitz, 1957; Powell and Powers, 1973). Lens regeneration could be prevented completely if the retina was permanently removed (Stone, 1958b). When iridocorneal complexes were transplanted on to the forehead they only regenerated a lens if the neural retina had regenerated either from the ciliary epithelium or the ora serrata (Zalokar, 1944a). Lens regeneration in the eye could be prevented by interposing a piece of pliofilm between neural retina and iris after lentectomy (Stone, 1958a,b). Fischel (1897) and Wachs (1914) grafted pieces of dorsal iris into the vitreous chamber of lentectomized eyes; lenses regenerated from the host iris and equally well from the grafted iris. Since no blood vessels nor nerves were present in these grafts, it was possible for Wachs to conclude that the stimulus for lens regeneration was propagated through the vitreous humour. Furthermore, Wachs (1914) noticed that the lens fibers developed on the side of the graft which was facing the neural retina, independently of the original orientation of the graft. When the normal or lens regenerating iris was cut out and reimplanted inside out in the adult *Notophthalmus viridescens* (Stone, 1954; Reyer, 1974, 1977b) or the developing lens was rotated inside out in the chick embryo (Coulombre and Coulombre, 1963), the lens now developed lens fibers on its new retinal side. In the eye with intact lens, iris pieces grafted into the anterior chamber cannot develop a lens, but regenerate small lenses in the posterior chamber (Uno, 1943; Takano et al., 1958). Similar results were obtained when embryonic lens vesicles of *Ambystoma punctatum* were implanted instead of irises (Reyer, 1959, 1966). As judged by autoradiography, the protein precursor ^{3}H-leucine is incorporated into the neural retina equally well in intact and lentectomized eyes; Gulati and Reyer (1980) therefore suggested that the retinal stimulator may be produced constantly. Yamada et al. (1973) cultured the newt dorsal iris in close apposition to the neural retina of the larval frog (*Rana pipiens*), and lenses at various developmental stages were formed. Under these conditions, ventral irises can apparently also regenerate a lens although to a lesser extent (Yamada and McDevitt, 1974).

The presence of the primary adult lens in the host eye, in general, inhibits lens regeneration from grafted pieces of donor iris in the vitreous chamber (Stone, 1943). Attempts to extract lens regeneration inhibiting proteins from the lens have been made by Smith (1965), Moore and Williams (1977), and Williams and

Reynolds (1977). Blocks made of agar, polyacrylamide or starch were soaked in different lens protein fractions and implanted into lentectomized eyes. One or two protein fractions inhibited lens regeneration more frequently than unimpregnated control blocks. Although the role of the lens in the prevention of lens regeneration in the normal eye is not yet sufficiently established, there is reasonably good evidence for a stimulatory effect of the neural retina.

8.6 COMPETITION OF IRIS AND LENS FOR THE STIMULATOR FROM THE RETINA

Some modifications of Spemann's hypothesis (1905) have been suggested. If the retina constantly secretes a trophic substance metabolized by lens and iris, the full-grown lens may be more efficient than the iris, and metabolize (Reyer, 1954, 1966) or absorb (Zalokar, 1944a; Reyer, 1954) most of this compound. After lentectomy the iris would receive a much larger proportion of this substance, and could regenerate a new lens. Reyer (1966) and Gulati and Reyer (1980) also mentioned the possibility that the zonular fibers which are destroyed during lentectomy may be dense enough to act as a filter for the retinal substance. Uhlenhut (1919) ascribed such a filtering function to a "connective tissue membrane" which envelopes the iris epithelium. No experimental evidence is available, which would permit distinction between these modifications of Spemann's hypothesis, but the competition hypothesis is attractive because it suggests a simplified control mechanism.

8.7 BLOCK OF THE SECRETION OF A RETINAL STIMULATOR BY AN INHIBITOR FROM THE LENS

Another modification of Spemann's hypothesis (1905) has been suggested by Reyer (1962), Stone (1965), and Scheib (1965). According to these investigators, the retina would only secrete a lens regeneration stimulating substance if the lens is removed. When *Cynops* irises from intact eyes were grafted into nonregenerating *Hynobius* eyes 50 to 60 days delayed instead of immediately after lentectomy, the frequency of lens regeneration dropped from 50% to 11% (Amatatu and Fujita, 1941). This suggests that the concentration of the stimulating substance may not be constantly high in the eye. Unfortunately, very little information is available on the physiological changes in the neural retina during lens regeneration. Synthesis of ribonucleic acid increases in all layers of the neural retina after lens removal (DeVito and Connelly, in Yamada, 1977), but only during the first 3 days (Gulati, 1980). The alkaline phosphatase activity increases in the retina from days 8 to 14 after lentectomy (Pósalaky et al., 1951; Yamada, 1977). When compared to the normal synthesis in the retina, the overall protein synthesis decreases at day 1 after lentectomy, increases from days 2 to 18, and decreases from days 19 to 30 (Gulati, 1980). Synthesis of deoxyribonucleic acid within the neural retina is enhanced at 7 days after lentectomy (Reyer, 1971a), and is most elevated in the region of the ora serrata and in the innermost layer of the neural retina. It seems that this modification of Spemann's hypothesis is promising and merits further experimental work.

8.8 EXTRAOCULAR STIMULATION OF THE IRIS

If the dorsal iris of the newt is grafted under either the skin (Ikeda, 1935; Monroy, 1937; Stone, 1943; Reyer, 1966), the larval dorsal fin (Reyer, 1966), the fourth brain ventricle (Ikeda, 1936a) or the peritoneal cavity (Ikeda, 1935; Stone, 1943), it will remain pigmented (stage 1 of lens regeneration) or will depigment partially (stage 2); lens regeneration under these conditions has only been reported in a few cases. Irises grafted into the peritoneal cavity have given rise to small lens-like regenerates (Stone, 1958a), and small lens vesicles and dwarfed lenses with lens fibers have been reported from dorsal irises grafted under the skin or into the brain cavities (Reyer, 1977a). Uhlenhut (1919) was the first to explant the iris into organ culture; he observed depigmentation under these conditions. He used the frog *Rana pipiens*, a species which is now known to be incapable of lens regeneration in the adult stage. Zarnik (1928) cited by Zalokar (1944a), Eisenberg Zalik and Meza (1968), Eisenberg-Zalik and Scott (1969), Connelly et al., (1973), Yamada et al., (1973), and Yamada and McDevitt (1974) have obtained similar results with newt or salamander irises which can regenerate lenses. Eguchi (1967) and Stone and Gallagher (1958) did not observe any sign of depigmentation in culture. After culturing the irises for about a month, the latter authors reimplanted this tissue into the eye of host animals; some of these irises regenerated a lens. Yamada et al. (1973), and Yamada and McDevitt (1974) obtained lens regenerates from newt irises in organ culture up to stage 3 in one case. From these above mentioned results it seems that the dorsal iris has an intrinsic ability to form a lens, but that trophic stimulation is necessary for the realization of lens regeneration (Yamada, 1977).

Dorsal iris epithelial cells can be dissociated using a mixture of proteolytic enzymes and cultured under cell culture conditions (Horstman and Zalik, 1974). These cells are able to depigment, migrate and aggregate in dense clusters, which have been called lentoid bodies (Eguchi et al., 1974; Zalik et al., 1976), since they contain lens proteins as shown by immunofluorescence and immunoelectrophoresis (Eguchi et al., 1974; Abe and Eguchi, 1977). Alpha, β-, and γ-crystallins have not been detected after 30 days of culture, but all crystallins are present after 40 days, at the time at which lentoid bodies have developed (Eguchi, Abe and Watanabe, 1974). Fully pigmented cells may also form dense clusters (Zalik and Dimitrov, 1980) and can freely mix with depigmented lens cells. These results further substantiate the contention that dorsal iris cells contain an intrinsic program for cell type conversion and lens formation. However, there are important differences in the behaviour of these cells when compared to that in the eye. Most important, ventral iris cells, which have very low lens formation capacity in the eye or in organ culture, can form lentoid bodies in cell culture just as easily as dorsal iris cells (Eguchi, Abe and Watanabe, 1974). The lentoid bodies formed under cell culture conditions are not organized into regions of lens epithelium and lens fibers; cells simply pile up on top of each other, and are not aligned (Zalik and Dimitrov, 1980). Lastly, lentoid body formation in cell culture seems to be slightly slower than lens regeneration in the eye (Zalik and Dimitrov, 1980; Abe and Eguchi, 1977). Zalik and Dimitrov (1980) reported that lentoid bodies did not appear before day 42 after explantation under their conditions; however, Eguchi (1979) claims that lentoid bodies formed as early as after 35 days.

Lenses regenerated from whole dorsal irises maintained outside of the eye are rare and rudimentary. Organ cultures of dorsal irises with spleen, lung, or skeletal muscle did not have higher lens regeneration frequencies than their controls (Connelly et al., 1973). However, two extraocular tissues have been discovered which can stimulate lens regeneration outside of the eye, namely regenerating limb blastemas with spinal nerves (Reyer et al., 1973) and the pituitary gland (Zalokar, 1944b). When dorsal irises were inserted in 8 to 20 day old blastemas of the regenerating forelimb of the newt, lens vesicles, deformed lentoids and lenses up to stage 9 developed after 20 days in 43 to 71% of the cases. However, in limb regenerates denervated at the time of implantation the frequency of lens regeneration was only 14%. When implanted into eyes without lens and neural retina spinal ganglia of regenerating limbs stimulated lens regeneration from dorsal iris with regenerates reaching up to stage 4 (Powell and Powers, 1973).

Zalokar (1944a,b) cultured whole lentectomized eye balls in which the neural retina tended to degenerate. Such eyes only regenerated lenses if a pituitary gland was inserted into their posterior chambers. Lenses also regenerated with a frequency of up to 96% and up to stage 10 (Yamada et al., 1973) if iridocorneal complexes, iris rings (Connelly et al., 1973), or isolated dorsal iris epithelia (Yamada et al., 1973) were cultured with the pituitary gland in close apposition. Powell and Segil (1976) and myself (unpublished observation) obtained a high frequency of regeneration of small secondary lenses in nonlentectomized eyes *in vivo* when pituitary glands were inserted into their anterior chambers. If the pituitary gland is removed in newts prior to lentectomy, lens regeneration will still take place (Nikitenko, 1940; Schotté and Murphy, 1953), but it is slightly delayed for a few days (Stone, 1957; Takano, 1962; Connelly et al., 1973; Stone and Steinitz, 1953b). Removal of the thyroid gland prior to lentectomy has no direct effect on lens regeneration (Lenhard, 1928; Schotté and Murphy, 1953; Stone and Steinitz, 1953b). It can be concluded that although two extraocular tissues can stimulate lens regeneration, they are not necessary for lens regeneration in the eye *in vivo*.

9. PURPOSE OF THIS THESIS

In view of the effect of the pituitary gland on lens regeneration, it appeared worthwhile to test whether any pituitary hormone preparation could stimulate lens regeneration. The organ culture system appeared most suitable to answer this question, since systemic influences could be ruled out. In addition, it was hoped to gain some insight into the mode of action of effective hormones by observing growth rate, and cell type transformation of dissociated dorsal iris epithelial cells in culture.

MATERIALS AND METHODS

1. PREPARATION AND MAINTENANCE OF CULTURES

1.1 MATERIALS

Modified amphibian Ringer's solution (Cuny and Zalik, 1981).

6500 mg NaCl
140 mg KCl
120 mg $CaCl_2$
100 mg $MgSO_4$
1800 mg D-glucose
200 mg gentamicin sulfate (Schering or Sigma)

$$\left.\begin{array}{r}\text{90 mg } NaH_2PO_4\\ \text{620 mg } Na_2HPO_4\end{array}\right\}\ \text{0.005 M phosphate buffer}$$

in 1 l of distilled water, pH 7.6. $CaCl_2$ must be dissolved before phosphate is added.

Calcium and magnesium free amphibian Ringer's solution (Horstman and Zalik, 1974).

6500 mg NaCl
140 mg KCl
1800 mg D-glucose
200 mg gentamicin sulfate (Schering or Sigma)

$$\left.\begin{array}{r}\text{90 mg } NaH_2PO_4\\ \text{620 mg } Na_2HPO_4\end{array}\right\}\ \text{0.005 M phosphate buffer}$$

in 1 l of distilled water, pH 7.6.

Dissociation medium for mixed dorsal iris stromal and epithelial cells (Dimitrov, unpublished).

5 parts of calcium and magnesium free amphibian Ringer's
  solution containing 0.4% (w/v) trypsin (Difco 1:250, 0152-15)
  and 0.01% (w/v) deoxyribonuclease I (Sigma D-0751)

1 part of calcium and magnesium free amphibian Ringer's
  solution containing 0.08% (w/v) collagenase (Sigma C-0130)

Solutions were made up the day before and stored in the refrigerator overnight and mixed immediately before use, adjusted to pH 7.4, and sterilized by sterile filtration. In one experiment a pure population of iris epithelial cells was prepared. In this case the dissociation medium was made up and mixed immediately before use, and adjusted to pH 7.2.

Cell culture medium (Horstman and Zalik, 1974).

50% Leibovitz L15 Medium with L-glutamine (Grand Island H-13)
50 μg/ml gentamicin sulfate (Sigma)
10% dialyzed fetal calf serum (Grand Island 614) with the salt
  concentration adjusted to that of modified amphibian
  Ringer's solution
40% distilled water

pH 7.6. Sterilized by sterile filtration, stored at 5°C.

Organ culture medium (Cuny and Zalik, 1981).

50% or 60% Medium 199 with Earle's salts (Grand Island E-11)
  and 2.2 gr/l $NaHCO_3$
50 μg/ml L-ascorbic acid

50 μg/ml gentamicin sulfate (Schering or Sigma)

10% dialysed fetal calf serum (Grand Island 614) with the salt concentration adjusted to that of modified amphibian Ringer's solution

30 to 40% distilled water

Fetal calf serum was dialyzed (24 hr, 5°C) in Spectrapore 2 bags (Spectrum Medical Industries) with a molecular weight cut-off at 12×10^3 to 14×10^3 daltons against 2 changes of 40 times its volume of distilled water. The final pH of the culture medium was 7.6. L-ascorbic acid was routinely added to the first series of cultures because it had been reported to stimulate lens and pigment cell differentiation in cultured embryonic neural retina cells of the chick embryo (Itoh, 1976). In a later series of experiments I found that this compound had no effect on cultured newt iris epithelial cells; this was also confirmed by Connelly (1980). Therefore, it was omitted from the culture medium.

1.2 EXPLANTATION OF DORSAL IRISES AND PITUITARY GLANDS

Adult newts (Lee's Newt Farm, Tennessee), kept at 12°C in dechlorinated water, were washed in potassium permanganate (10 mg/1, 1 hr), anesthetized in ethyl-m'-aminobenzoate methane sulfonate (Sandoz; 1 gr/1, 20 min) and their heads were quickly washed in 70% ethanol and sometimes in sterile distilled water. All further steps were performed with the help of a dissecting microscope under sterile conditions in a plexiglass hood irradiated for 30 min with ultraviolet light prior to the operation. Tools were sterilized in 70% ethanol, and sterile plastic petri dishes

(Falcon 1007; 60 x 15 mm) were used, filled with 10 ml of modified amphibian Ringer's solution.

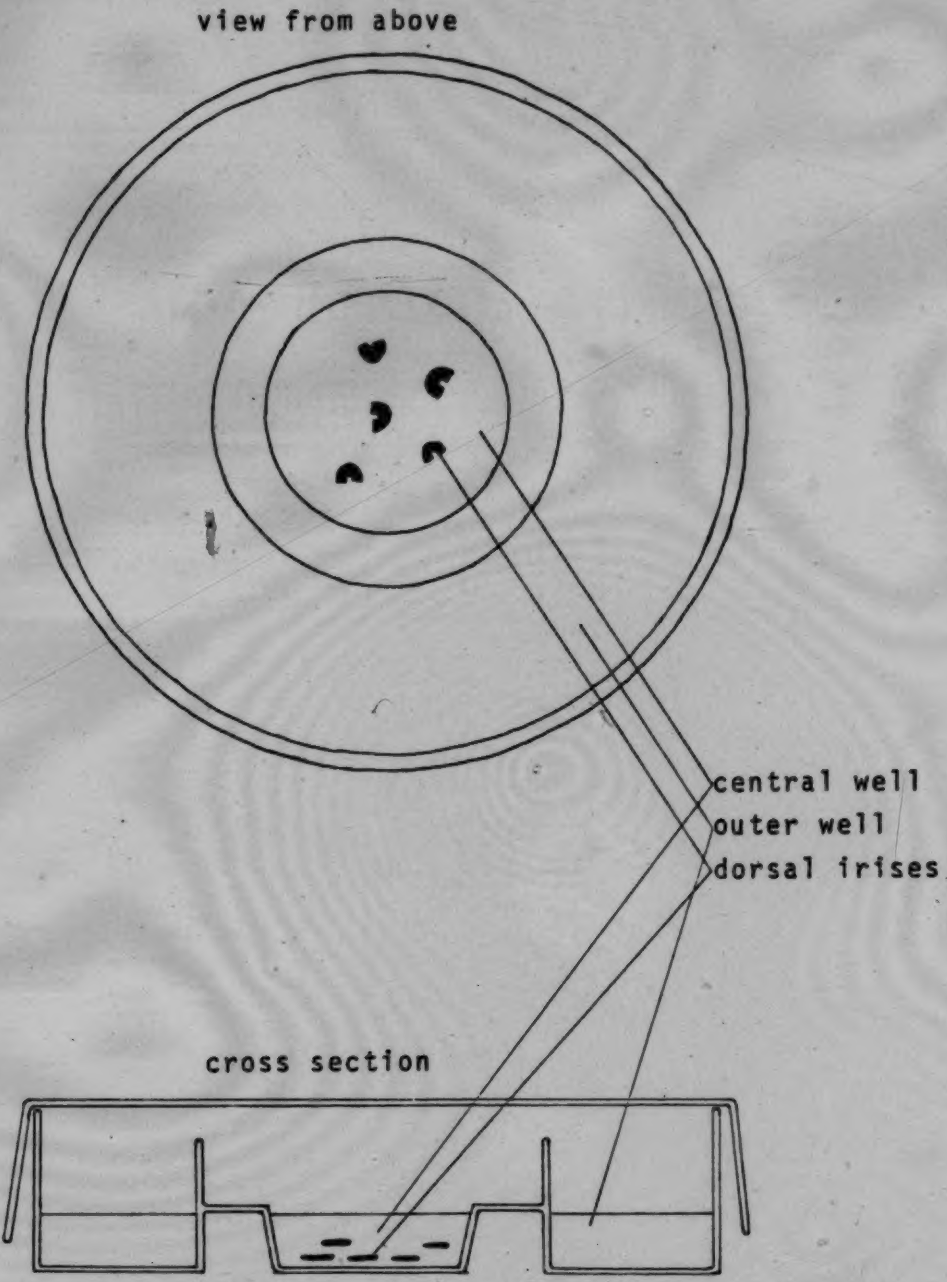
For the removal of the iris, the lens was gently pressed out of the eye through a horizontal incision in the cornea made with a straight scalpel blade. The dorsal half of the cornea without the sclerocorneal junction was then cut out together with the adhering iris and transferred into modified amphibian Ringer's solution, which was sometimes cooled on ice. The iris tissue was separated from the corneal tissue using scalpel blades, washed once in modified amphibian Ringer's solution, and transferred into either organ culture medium or cell dissociation medium depending on the experiment. It seemed important to excise the iris tissue as fast as possible. Therefore, in later experiments the washing step in sterile distilled water was omitted. Keeping the irises in cooled modified amphibian Ringer's solution improved subsequent cell viability, but the time of excision was slightly extended.

Pituitary glands were obtained as follows: The head of anesthetized newts without lower jaws was cut from the body. It was then cut sagittally into two halves. The slightly bigger half contained the pituitary gland behind the optic chiasma. The pars distalis was removed together with the pars intermedia, and the latter was not dissected away.

1.3 MAINTENANCE OF ORGAN CULTURES

In each experiment, 20 explants per treatment group were maintained for 20 days. Irises were cultured in 0.3 ml of medium

Figure 4. Schematic sketch of the organ culture plastic dish (Falcon (Stevens) 3037; 60 x 15 mm).X 1.7. Five ml of sterile distilled water were added to the outer well; 0.3 ml of organ culture medium were added to the central well, where the irises were cultured.


view from above
central well
outer well
dorsal irises
cross section

present in the central well of organ culture plastic dishes (Falcon (Stevens) 3037; 60 x 15 mm); dishes were gently swirled once a day to prevent attachment of the tissues. Five ml of sterile distilled water were added to the outer well (Figure 4) to maintain humidity. The culture medium was changed daily. All solutions and media were sterilized by filtration through Millipore filter GS 46 μm pore size, in a Sartorius filter holder under vacuum suction. Organ cultures were maintained at 23°C in a ventilated humidified atmosphere (90 to 100% saturation) containing approximately 1% CO_2. Dialysed sera were kept frozen in 20 ml aliquots at -20°C, and sterile complete organ culture media were stored at 5°C for not more than 14 days.

Hormone preparations were added to the culture medium as follows. Bovine thyrotropin (NIH, TSH-B8 and Sigma, TS-10), ovine thyrotropin (NIAMDD, oTSH-9), human thyrotropin (CalBiochem, hTSH), bovine follitropin (NIH, FSH-B1), bovine lutropin (NIH, LH-B10), and bovine pituitary fibroblast growth factor (Collaborative Research, FGF-40002) were directly dissolved in complete organ culture medium at pH 7.6. Bovine prolactin (NIH, PRL-B4) and somatotropin (NIH, GH-B18) were first dissolved in triple distilled water at pH 9.5, and the pH was then adjusted to 7.6. Both hormone solutions were combined with undiluted organ culture medium at a ratio of 2:3 (v/v) to the desired hormone concentration. Porcine adrenocorticotropin (Sigma, A-6002) was dissolved directly in complete organ culture medium at pH 7.0, and the pH was then adjusted to 7.6. After sterile filtration, aliquots of hormone supplemented media were stored frozen in liquid nitrogen.

1.4 DISSOCIATION OF DORSAL IRIS CELLS

Excised dorsal irises were washed once in complete modified amphibian Ringer's solution, transferred to a different dish (Falcon 1007, 60 x 15 mm) and briefly washed once in calcium and magnesium free amphibian Ringer's solution.

To obtain reasonably pure preparations of dorsal iris epithelial cells, dorsal irises were transferred to sterile 10 ml test tubes with loosely fitting screw caps (Pyrex 9826) filled with 6 ml of dissociation medium. Tubes were incubated at 31°C on a gyrotory shaker (New Brunswick Scientific) at a speed of 170 r.p.m. for a total of 70 min with a change of dissociation medium after 30 min of incubation. Irises were then pipetted gently at room temperature until the black iris epithelial cells had dissociated. The still intact iris stromas were then removed with a pipette and discarded.

An alternative method has been used to obtain mixed dorsal iris stromal and epithelial cell populations. Dorsal irises were transferred into sterile 25 ml beakers covered with aluminum foil, and 6 ml of dissociation medium were added. The incubation period was for 90 min at 31°C with one change of medium after 30 min in a gyrotory shaker at 90 r.p.m. Total dissociation was achieved by gentle pipetting of irises at room temperature.

After dissociation, complete cell culture medium was added slowly and mixed well with the cell suspension in the dissociation medium at a ratio of 1:1 (v/v). The cell suspension was centrifuged at 250 x g at 22°C for 5 min. Three quarters of the supernatant were discarded, the cells were resuspended in the remaining quarter by gentle pipetting and complete cell culture medium was again added and the mixture centrifuged. This procedure was repeated twice. Cell suspensions were divided into aliquots and were seeded into 4 to 8 cell culture plastic dishes (Falcon 3001; 35 x 10 mm).

1.5 MAINTENANCE OF CELL CULTURES

Cells were allowed to attach for 14 days in 2.5 ml of complete cell culture medium. Thereafter, the medium could be changed every 7 days. Cultures were maintained for up to 110 days. One tenth (0.25 ml) of the total volume of medium in a culture dish containing 10 times concentrated hormone preparations was added every 2nd day. Therefore, the hormone concentrations were not constant in the cell culture experiments. Hormone activity slowly decreases in aqueous solution at room temperature and addition of fresh hormone preparation every 2nd day seemed necessary. The cell culture dishes were kept in a plexiglass culture box at room temperature. Hormone containing media were stored frozen in aliquots in liquid nitrogen until used.

2. HISTOLOGICAL PREPARATION

2.1 MATERIALS

Bouin's fixative(Conn et al., 1960)

75% picric acid, saturated aqueous solution

25% formalin, saturated aqueous solution of formaldehyde

5% acetic acid, glacial

pH 7.0.

Ehrlich's hematoxylin (Conn et al., 1960)

100 ml 95% ethanol with 2 gr hematoxylin (Fisher)

100 ml distilled water with 3 gr aluminum potassium sulfate or aluminum ammonium sulfate

100 ml glycerin

10 ml acetic acid, glacial

ripened with 0.2 gr $NaIO_4$.

Alcoholic eosin (Conn et al., 1960)

95% ethanol with 1% (w/v) eosin Y (Fisher)

ripened for 2 months (colour changes from green to yellow).

Phosphate buffered saline, 0.1 M

8.0 gr NaCl

3.0 gr NaH_2PO_4

21.2 gr Na_2HPO_4

in 1 l distilled water, pH 7.4.

Primary antibody solution.

0.3 ml rabbit anti-frog γ-crystallin antiserum, anti-α,β,γ-crystallin antiserum, and anti-pituitary crude glycoprotein extract antiserum diluted with 0.3 ml of 1% (w/v) leopard frog (*Rana pipiens*) tissue powder in 0.1 M phosphate buffered saline and incubated for 30 min at room temperature.

5.4 ml 0.1 M phosphate buffered saline

The frog tissue powder was prepared as follows: Whole frogs without their lenses and pituitary glands were homogenized in distilled water in a Sorvall blender, lyophilized, and stored at 5°C.

Secondary antibody solution.

0.5 mg/ml fluorescein conjugated goat anti-rabbit immunoglobulin G, as powder (Microbiological Associates 51-781) or in solution (Miles 65-173) diluted with 0.1 M phosphate buffered saline to a final dilution of 1:20.

2.2 TISSUE PROCESSING

Cultured tissues were fixed in Bouin's fixative for 2.5 hr, and were washed for 1 hr in several changes of 0.1 M phosphate buffer, pH 7.4, at room temperature. They were then dehydrated in graded ethanol and xylene on crushed ice, transferred through 3 changes of paraplast (Lancer, Sherwood) at 56°C for 3 hr, and embedded (Lipshaw plastic molds), and sectioned at 7 μm on a Spencer AO 280 microtome. Sections were rehydrated in xylene and graded ethanol on ice and subjected to immunofluorescent staining or directly to hematoxylin-eosin staining.

2.3 HEMATOXYLIN-EOSIN STAINING AND MOUNTING

Tissue sections were washed in deionized water, and stained for 1 to 5 min in Ehrlich's hematoxylin. They were washed in deionized water, contrasted in tap water, and gradually brought up to 70% ethanol. They were stained for 1 to 5 min in alcoholic eosin, rinsed in 70% ethanol and dehydrated in graded ethanol and xylene. The slides were mounted in DPX (BDH Chemical Co.). Cell cultures were fixed directly in their plastic culture dishes, stained, and mounted in Aquamount (E. Gurr LTD,London).

2.4 IMMUNOFLUORESCENT STAINING

The indirect immunofluorescence method was used. Tissue sections were deparaffinized for 20 min in xylene on ice; they were then hydrated in a graded ethanol series, and washed in 0.1 M phosphate buffer, pH 7.4, at 22°C, for 10 min. This was followed by two 30 min changes of 0.1 M phosphate buffered saline. Fixed cell cultures were directly washed in 0.1 M phosphate buffer and processed further like the tissue sections.

All further steps were performed at room temperature. Tissue sections or cell culture dishes were coated with primary antibody solution, and incubated for 2 hr in moist petri dishes at room temperature. Slides or cell culture dishes were then washed in 3 changes of 0.1 M phosphate buffered saline for 1 hr and were then coated with secondary antibody solution. The incubation time was again 2 hr. After washing for 1 hr with 3 changes of 0.1 M phosphate buffered saline, slides or cell culture dishes were temporarily mounted in glycerin. These preparations could later be stained with hematoxylin-eosin and permanently mounted in DPX or Aquamount.

3. PREPARATION OF ANTIBODIES

3.1 MATERIALS

Buffer system for ion exchange chromatography (McDevitt et al., 1969). (Figure 5).

(1) 0.005 M phosphate, pH 7.2

(2) 0.0075 M phosphate, pH 6.5

(3) 0.01 M phosphate, pH 6.0

(4) 0.02 M phosphate, pH 5.7

(5) 0.02 M phosphate, pH 5.7, 0.1 M NaCl

(6) 0.1 M phosphate, pH 5.7, 0.3 M NaCl

Pituitary glycoprotein extraction medium (Stockell Hartree, 1975).

42 ml of 10% (w/v) ammonium acetate previously adjusted to pH 5.1

28 ml of 96% ethanol

Barbital-acetate buffer, 0.05 M.

10.8 gr/l sodium diethylbarbiturate (Fisher)

8.6 gr/l sodium acetate

11.65 ml/l of 0.1 M HCl

pH 8.5.

Coomassie brilliant blue staining solution

908 ml 50% (v/v) methanol

92 ml acetic acid, glacial

2.5 gr/l Coomassie brilliant blue R powder (Sigma)

filtered through Whatman 3 filter paper.

Destaining solution

875 ml distilled water

75 ml acetic acid, glacial
50 ml 96% ethanol

Storage solution

520 ml distilled water
280 ml glycerin
200 ml 96% ethanol

3.2 PURIFICATION OF LEOPARD FROG γ-CRYSTALLIN

The purification of γ-crystallin from the lens of the frog (*Rana pipiens*) has been reported by McDevitt (1967), McDevitt et al. (1969), and Nöthiger et al (1971) and from the lens of the newt (*Notophthalmus viridescens*)by Takata et al. (1965), McDevitt et al. (1969) and Nöthiger et al. (1971). Lenses were excised from previously frozen adult leopard frogs (*Rana pipiens*) and were stored at -12°C. They were homogenized on ice in 0.005 M phosphate buffer at pH 7.0. For extraction, the homogenate was allowed to stand overnight at 5°C and was then centrifuged at 6300 x g for 1 hr at 5°C. The supernatant was dialyzed for 2 days against 2 changes of 2 l of distilled water and lyophilized. The resulting powder is referred to as the total α, β, γ-crystallin extract.

The α, β, γ-crystallin powder was dissolved in 10 ml of 0.005 M phosphate buffer, pH 7.2. Anion exchange chromatography was performed on a 7 gr diethylaminoethylcellulose (Whatman DE 32) column (3.0 x 5.4 cm) at 5°C with a bed volume of 38 ml. The diethylaminoethylcellulose had been precycled in 50 ml of 0.5 M

HCl, washed in distilled water, and cycled in 0.5 M NaOH. It was washed in distilled water, and equilibrated in 2 changes of 50 ml of 0.005 M phosphate buffer, pH 7.2. Slowly sedimenting fines of the ion exchanger were decanted with the supernatants. The protein concentration in the effluent was monitored using an ultraviolet absorption detector (Instrumentation Speciality Co. Model UA 2) operated at 280 nm, with an automatic recorder. The buffer flow was regulated with a polystaltic pump (Buchler Instruments), and fractions were collected on an automatic fraction collector (LKB Ultrorac Type 7000) programmed for 90 drops (= 6.43 ml) per fraction. The buffer level on top of the column was lowered to the diethylaminoethylcellulose surface before adding new buffer. The buffer system is described in chapter 3.1 and the elution pattern is shown in Figure 5 (a). The nonadsorbed cationic fraction (0 to 200 ml effluent) contained the largest amount of γ-crystallin. Only this first γ-crystallin peak was dialysed against distilled water (2 changes of 2 l, 16 hr each), lyophilized and purified further.

The crude γ-crystallin powder was redissolved in 5 ml of 0.05 M Tris-HCL buffer, pH 7.2. It was centrifuged (6300 g, 1 hr, 5°C) and the supernatant was applied to an 86 x 2.6 cm Sephadex G-100 column (Pharmacia, void volume 125 ml). This column was prepared by previous swelling of 27 gr of Sephadex beads which were then equilibrated in 1 l of 0.05 M Tris-HCL buffer, pH 7.2. Gel filtration chromatography was performed at 5°C. The flow rate of buffer was controlled by the polystaltic pump, the protein concentration

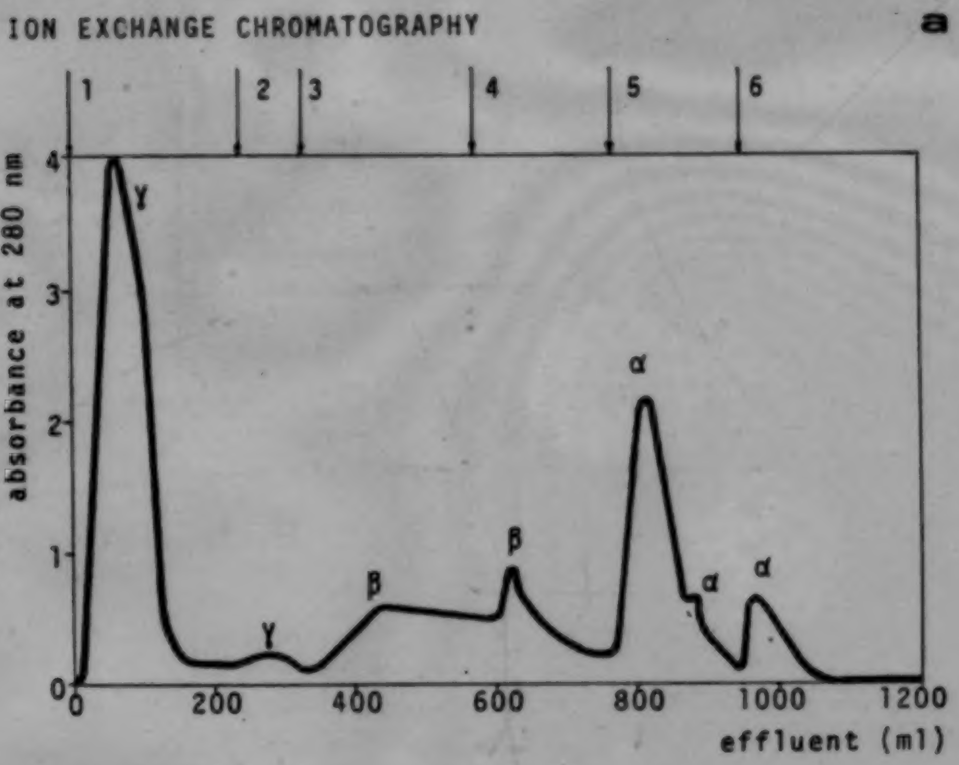
Figure 5. Purification of γ-crystallin of the leopard frog (*Rana pipiens*).

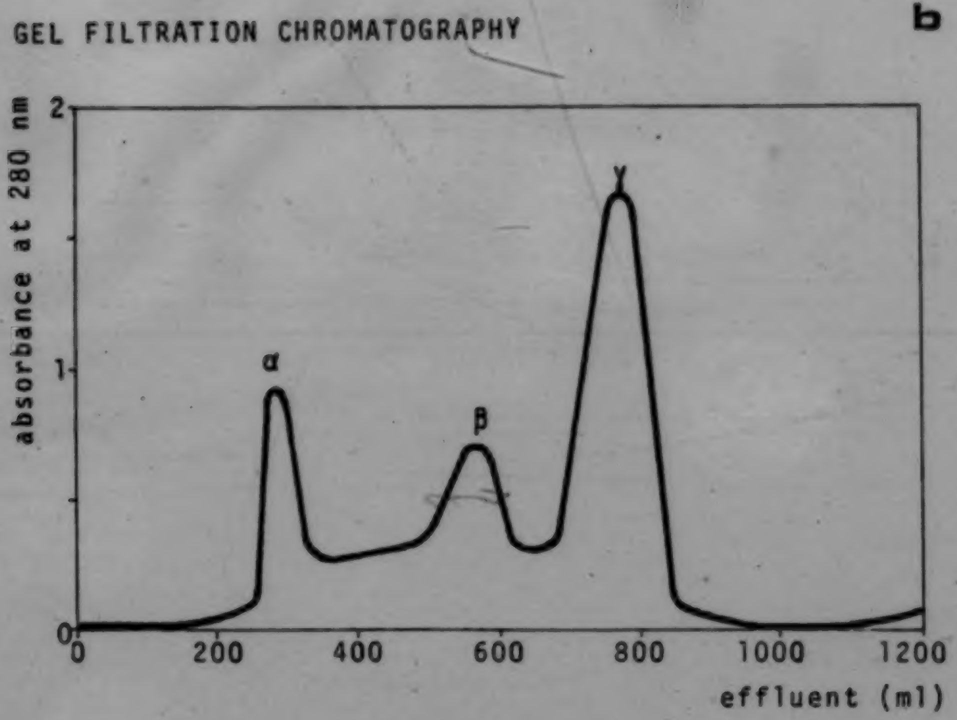
a. Ion exchange chromatography: 7 gr diethylaminoethylcellulose, column 38 ml, phosphate buffer. The buffer was changed when indicated on top of the graph (1 to 6):

(1) 0.005 M phosphate, pH 7.2
(2) 0.0075 M phosphate, pH 6.5
(3) 0.01 M phosphate, pH 6.0
(4) 0.02 M phosphate, pH 5.7
(5) 0.02 M phosphate, pH 5.7, 0.1 M NaCl
(6) 0.1 M phosphate, pH 5.7, 0.1 M NaCl

The protein concentration in the effluent was monitored with an ultraviolet detector. The nonadsorbed cationic fraction eluted first and was composed mainly of γ-crystallin.

b. Gel filtration chromatography: 27 gr Sephadex G-100, column 86 x 2.6 cm, 0.05 M Tris-HCL buffer, pH 7.2. The protein concentration in the effluent was monitored with an ultraviolet detector. The largest molecular size fraction eluted first.


ION EXCHANGE CHROMATOGRAPHY
a
1
2
3
4
5
6
absorbance at 280 nm
0
1
2
3
4
γ
γ
β
β
α
α
α
0
200
400
600
800
1000
1200
effluent (ml)


GEL FILTRATION CHROMATOGRAPHY
b
absorbance at 280 nm
0
1
2
α
β
γ
0
200
400
600
800
1000
1200
effluent (ml)

in the effluent was monitored and fractions were collected as described above. The elution pattern can be seen in Figure 5 (b). The smallest molecular size fraction, which eluted last (680 to 850 ml effluent) contained the γ-crystallin. The pooled γ-crystallin fractions were dialyzed at 5°C against 2 changes 6 hr each of 500 ml of distilled water, centrifuged (6300 g, 1 hr, 5°C) and the supernatant was lyophilized. The yield from 23 frog lenses was 48 mg of γ-crystallin.

3.3 PREPARATION OF CRUDE GLYCOPROTEIN EXTRACT FROM LEOPARD FROG PITUITARY GLANDS.

Koenig and King (1950) introduced the ammonium acetate-ethanol extraction technique to extract pituitary glycoprotein hormones. Although some proteins contaminate these extracts, Stockell Hartree (1966) found no protein hormones such as somatotropin or prolactin, in the preparation of extracted glycoproteins. Pituitary glycoproteins of many vertebrate species (Stockell Hartree, 1975), including bullfrog (*Rana catesbeiana*) (Licht and Papkoff, 1974) and tiger salamander (*Ambystoma tigrinum*) (Licht, Farmer and Papkoff, 1975), are extracted in high yield with this method and retain their biological activities.

Six hundred pituitary glands from leopard frogs (*Rana pipiens*) were excised from previously frozen animals and immediately dropped into a container submerged in acetone-dry ice at -63°C. They were extracted with several changes of cold acetone at 5°C and homogenized in acetone. The acetone powder was allowed to sediment and was dried at 5°C exposed to the air in a petri dish for 3 days. It was further dried in the vacuum at 22°C for 1 hr. The acetone powder was extracted twice overnight at 5°C with slow stirring in pituitary glycoprotein extraction medium. After centrifugation at 1400 g for 30 min, the supernatant was saved and the residue was reextracted with fresh extraction medium. After the second extraction and centrifugation, the residue was washed twice with 30 ml of acetone, air dried at 5°C and stored in the vapor phase above the liquid nitrogen. From the combined supernatants, the crude glycoprotein fraction could be precipitated by adding within 30 min twice the volume of cold 96% ethanol with slow stirring. Precipitation was allowed to occur for two days at 5°C. The mixture was centrifuged, and the supernatant was discarded. The latter contains small peptides, such as adrenocorticotropin or melanocyte stimulating hormone (Stockell Hartree,1966) The precipitate, which mainly contains glycoproteins (Stockell Hartree, 1966), was dialyzed at 5°C in Spectrapore 1 dialysis bags (Spectrum Medical Industries, molecular weight cut-off at 6 x 10^3 daltons) against two changes of 2 l each of distilled water at pH 5.1 for 2 days. The pH of the retentate was readjusted to 7.6 and the fraction was lyophilized. These conditions were used by Reichert (1975) during his purification of glycoprotein hormones. The yield of 600 frog pituitary glands was 9.1 mg of crude glycoprotein powder.

3.4 IMMUNIZATION OF RABBITS

Virgin, 5 months old female San Juan rabbits were used. Two days before the injection, they were starved overnight to decrease their blood lipid level. In the restrainer box, blood (2 to 4 ml) was taken with a 23 gauge 1 needle from their ear vein, which was previously dilated by xylene application and washing with water. Test blood was collected in a Sorval glass centrifuge tube and allowed to clot for 1 hr at 22°C. The clot was periodically separated from the glass wall with a blunt glass rod. The blood sample was then centrifuged in a clinical centrifuge at 250 x g and 22°C for 10 min. The clear serum above the clot was pipetted into plastic capsules and kept frozen above liquid nitrogen (about -60°C) until used.

On the next day after bleeding, injections of antigen were made hypodermally into the back of the rabbit. For the first injection, 0.9 ml of complete Freund's adjuvant (Difco) were mixed with 0.1 ml of saline (8.5 gr NaCl/1 H_2O) containing the antigen. For subsequent injections, incomplete Freund's adjuvant (Difco) was used instead of complete adjuvant. The antigen injection schedules are shown in Table 2.

The rabbits were bled by heart puncture. The blood was drawn into a 50 ml syringe equipped with an 18 gauge 1.5 needle, and allowed to clot for 1 hr at 22°C in polyethylene centrifuge tubes (Sorvall). The clot was separated from the plastic surface with a blunt glass rod twice. The clot was allowed to contract for 24 hr at 5°C. The serum and clot were centrifuged in a clinical centrifuge at 250 x g at 22°C for 10 min. The clear supernatant serum was removed and centrifuged once more. This final supernatant was stored frozen in 0.3 ml

Table 2. Antigen injection schedules for the preparation of the different antisera.

γ-crystallin	α,β,γ-crystallin	pituitary glycoprotein
day 1: 5 mg	day 1: 10 mg	day 1: 1 mg
day 21: 5 mg	day 21: 10 mg	day 28: 1 mg
day 42: 10 mg	day 42: 20 mg	day 49: 2 mg
day 65: 10 mg	day 65: 20 mg	day 70: 2 mg
day 84: sacrificed	day 84: sacrificed	day 90: sacrificed

aliquots in plastic capsules above liquid nitrogen (about -60°C) until used.

3.5 POTENCY AND SPECIFICITY TESTS FOR ANTISERA.

3.5.1 Ring Test. 40 µl of antiserum (anti-γ-crystallin, anti-α, β, γ-crystallin or anti-pituitary glycoprotein antiserum) were diluted to concentrations of 1, 1/2, 1/4, 1/8, 1/16, and 1/32 of serum by serially pipetting 20 µl of serum into consecutive wells of a microbiological plastic test plate (Fisher 14-247-75), which contained 20 µl of saline (8.5 gr/l NaCl). These 6 serum dilutions were filled into the bottom of 6 microtest tubes (2 x 40 mm) at 22°C. Twenty µl of antigen containing saline were carefully layered on top of each serum dilution. Antigen concentrations were kept constant in all test tubes, at 5 µg/µl. The development of antibody-antigen complex precipitates at the interface between the antigen solutions and the serum dilutions was recorded. The lower the serum concentration that could still form a precipitate, the stronger the antiserum. The anti-γ-crystallin antiserum reacted up to the dilution 1/16, the anti-α, β, γ-crystallin antiserum up to 1/32, and the anti-pituitary glycoprotein antiserum up to a 1/16 dilution

3.5.2 Immunoelectrophoresis. Glass slides (5 x 12 cm) were washed in detergent, rinsed, washed in diluted hydrochloric acid, rinsed and air dried. They were coated with a thin layer (2 ml) of 0.3% (w/v) agarose (purified,BioRad) in distilled water. The agarose was dried completely on the slide at 60^{0}C for about 1 hr. The second coat consisted of 10 ml of 1.44% (w/v) purified agarose in distilled water, mixed before cooling 1:1 (v/v) with twice concentrated barbital-acetate buffer. It was evenly spread with a warm glass rod and allowed to polymerize. A longitudinal central trough and two lateral wells were cut with a razor blade cutter (Gelman) and a well cutter (Gelman).

The electrophoresis was performed in an electrophoresis cell (Turner Associates Model 310) connected to a regulated power supply (Gelman Instrumentation Co. Model DeLuxe). The agar gel was connected to the buffer chambers by several layers of Whatman filter paper strips. Thirty μg of frog α, β, γ-crystallin were dissolved in 15 μl of barbital-acetate buffer and applied to both lateral wells on the agar slide. Electrophoresis was performed at 8.3 V/cm, 15 mA, and 5^{0}C for 3 hr. After electrophoresis, slides were placed into moist chambers and anti-γ-crystallin or anti-α, β, γ-crystallin antiserum was poured into the longitudinal trough. Immunodiffusion was allowed to occur for 48 hr at 5^{0}C and precipitation arches formed. Nonprecipitated antibodies and antigen were washed out in 0.1 M phosphate buffered saline, pH 7.2, for at least 48 hr at 5^{0}C. The saline was changed twice.

The agarose slides were washed with distilled water for 2 hr at 5^{0}C. A filter paper (Whatman) was moistened and placed on top of the agar gel, and the slide was dried completely at 37^{o}C. The filter paper was removed and the agarose slide was stained in Coomassie brilliant blue staining solution for up to 3 min. After overstaining, destaining (for several months) was done in a destaining solution to achieve proper contrast. Slides could be stored for photography in storing solution.

RESULTS

1. EFFECT OF NEWT PITUITARY GLAND

1.1 ORGAN CULTURE

1.1.1 Regeneration Stages. Lens regeneration stages attained by irises in organ culture were classified according to Sato (1940) as adapted for adult *Notophthalmus viridescens* by Yamada (1967). The criteria have been outlined in the introduction (pp. 27-33). Stages 1 and 2 are reversible cell activation stages undergone by dorsal as well as ventral iris, and do not necessarily indicate the onset of lens regeneration. For the purpose of statistical evaluation we only regarded stages 3 and higher as indicative of lens regeneration. To compare the frequency of lens regeneration in control and experimental treatment groups the 2 x 2 table Chi square test for independence was applied. Irises that had reached stage 3 or higher were regarded as regeneration positive, while irises which had only attained stage 1 or 2 were regarded as regeneration negative. In most of the experiments some of the irises were lost during the handling of the tissue and were not available for analysis.

In order to test whether dorsal iris sectors responded to the newt pituitary gland under the present culture conditions dorsal irises were cultured with this tissue. Under our conditions in six replicate experiments, lens regeneration occurred in 44% (7 of 16), 46% (6 of 13)(Figure 6), 44% (8 of 18), 50% (7 of 14), 53% (10 of 19) and 67% (10

Figure 6. Lens regeneration stages achieved by dorsal irises after 20 days of culture with or without a newt pituitary gland in close apposition. The line between lens regeneration stage 2 and 3 represents the border between specific and nonspecific lens regeneration stages as defined in the introduction (p. 33). The numbers above the histograms represent the number of individual irises at that particular regeneration stage. Twenty irises were cultured, but only the ones harvested are included in the statistical analysis. The frequency of specific lens regeneration stages (stages 3 and higher) is significantly higher in cultures supplemented with pituitary glands as compared to control cultures. The significance levels (Chi square) are: $^{***}p < 0.005$, and $^{o}p < 0.10$ (almost significant). The numbers at the left of each treatment group represent the number of irises in each group.

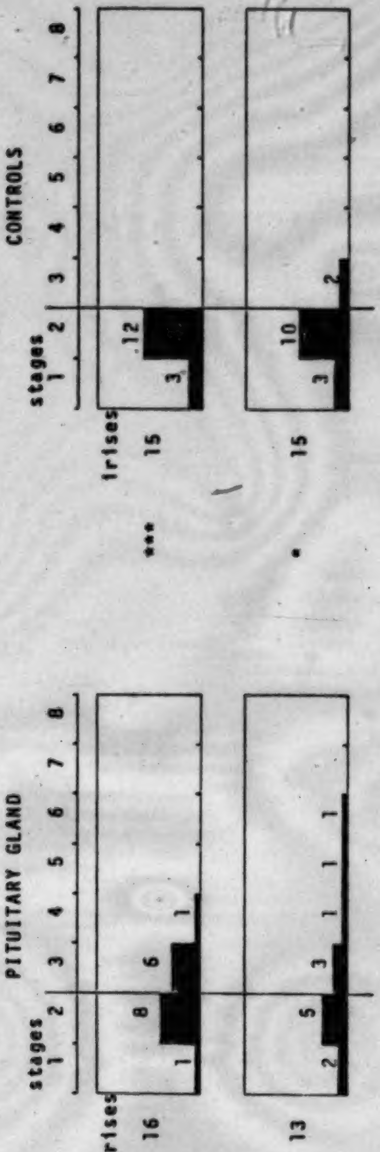

CONTROLS
PITUITARY GLAND
stages
1
2
3
4
5
6
7
8
Irises
15
15
16
13
12
3
10
3
2
***
*
1
8
6
1
2
5
3
1
1
1

Figure 7. Lens regeneration stages achieved by dorsal irises after 20 days of culture with or without a newt pituitary gland in close apposition. These cultures were run parallel with the various preparations of thyrotropin and fibroblast growth factor, as controls for favorable culture conditions (compare Figures 21, 24, and 46 to 51). The frequency of specific lens regeneration stages (stages 3 and higher) is significantly elevated in cultures with pituitary glands when compared to the controls (Chi square). The significance levels are: $***P < 0.005$, $**P < 0.01$, and $*P < 0.05$. The presence of γ-crystallin was confirmed by immunofluorescence in advanced lens regenerates and in a few early stages, on irises cultured with pituitary glands. (+) Faint fluorescent label in only 1 to 10 cells per lens regenerate, (++) strong fluorescence in 11 to 50 cells, (+++) strong fluorescence in over 100 cells. See Figure 6 for further explanation of the histograms.

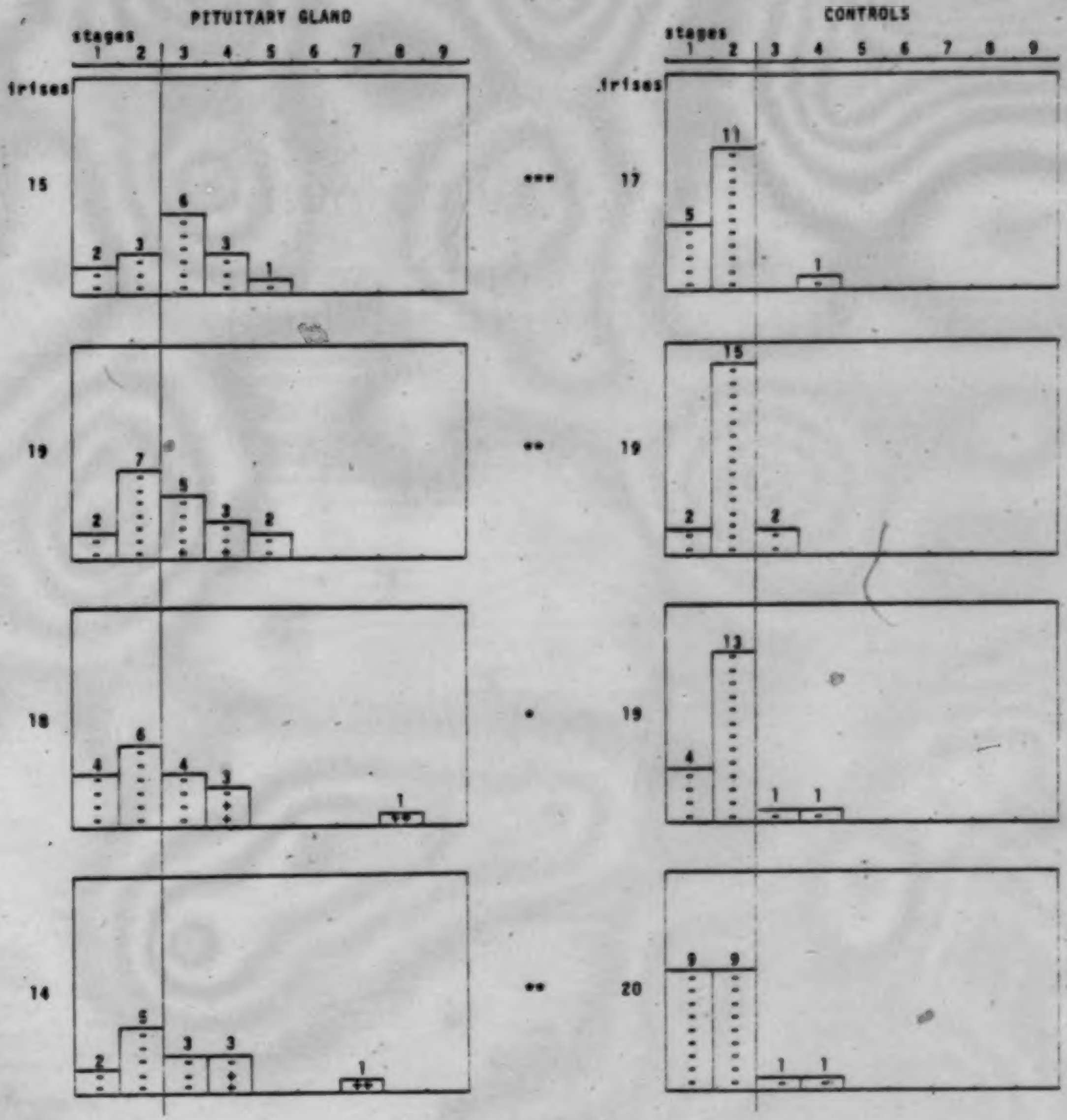
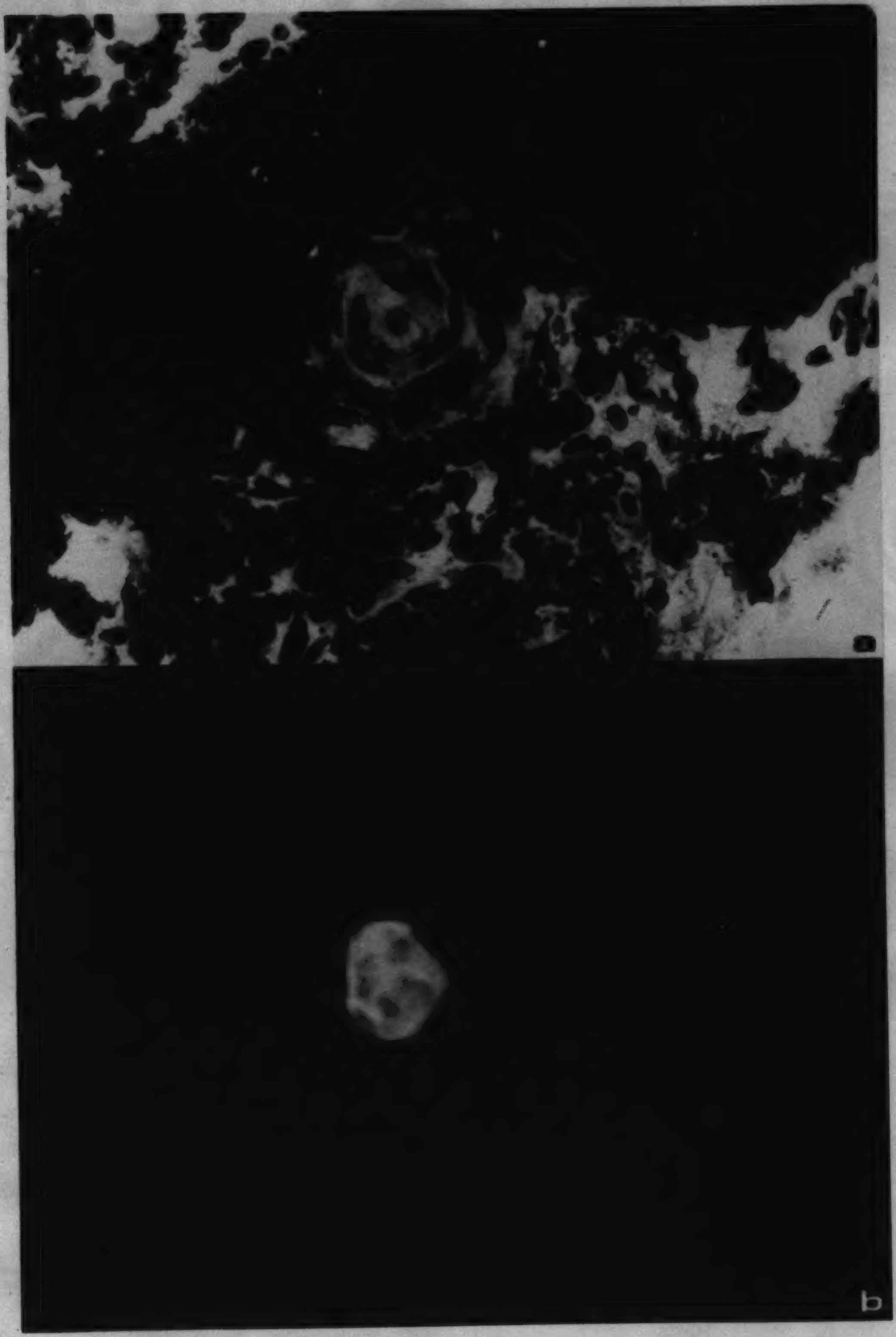

PITUITARY GLAND
CONTROLS
stages
1 2 3 4 5 6 7 8 9
irises
15
2 3 6 3 1
***
17
5 11 1
19
2 7 5 3 2
**
19
2 15 2
18
4 6 4 3 1
*
19
4 13 1 1
14
2 5 3 3 1
**
20
9 9 1 1

Figure 8. Gamma-crystallin accumulation in a stage 8 lens regenerate, which developed on a dorsal iris after 20 days of culture in the presence of a newt pituitary gland (below lens). (a) Stained transmission light micrograph, and (b) corresponding immunofluorescent light micrograph stained with anti-frog γ-crystallin antiserum. (a,b) X 225. Secondary lens fibers and lens epithelium-like cells totally surround the stained primary lens fiber core.


a
b

of 15)(Figure 7), as compared to none, 13% (2 of 15)(Figure 6), 6% (1 of 17), 10% (2 of 20), and in two experiments 11% (2 of 19) in the controls (Figure 7). The most advanced lens regenerates were lens vesicles (stages 4 and 5), lens vesicles showing the beginning of lens fiber development (stage 6) (Figure 6), lens vesicles with a primary lens fiber hillock (stage 7)(Figure 7), or regenerates with secondary lens fibers (stage 8)(Figures 7, 8). In the controls only depigmentation stage 2, or early lens vesicle formation (stage 4)(Figures 6, 7) were achieved. Thus, under these conditions irises were able to respond to the influence of the pituitary gland with a significantly augmented frequency of lens regeneration.

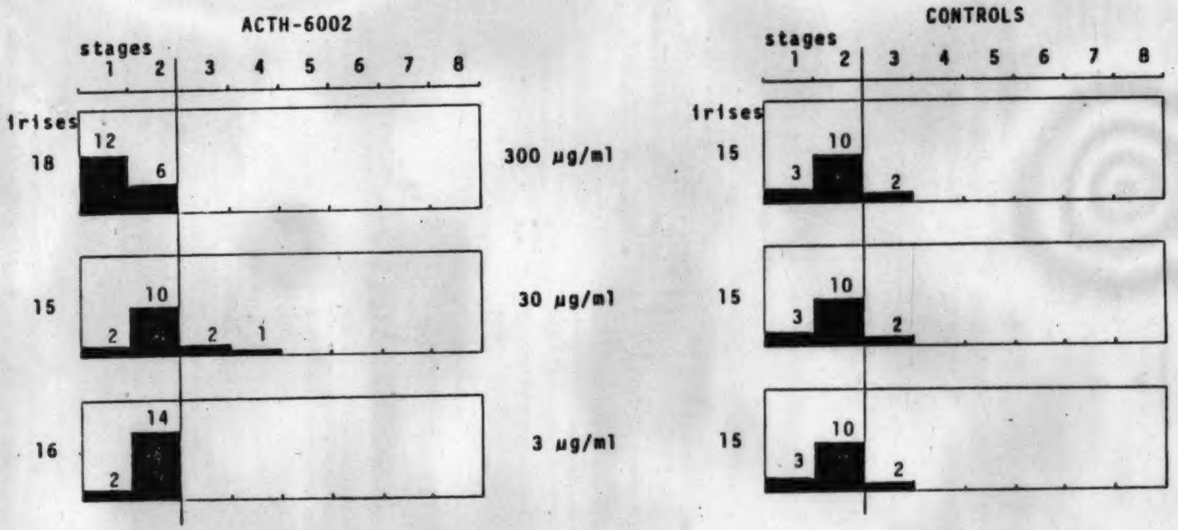
1.1.2 Presence of γ-Crystallin. As detected by immunofluorescence using anti-γ-crystallin antiserum γ-crystallin was present in regenerates at stage 6 and onwards where lens fiber differentiation was occurring (Figures 7, 8). Some earlier lens regenerates of stages 3 and 4 had occasionally a few γ-crystallin-positive cells.

2. EFFECT OF PORCINE ADRENOCORTICOTROPIN PREPARATION ACTH-6002

2.1 ORGAN CULTURE

2.1.1 Regeneration Stages. At concentrations between 300 and 3 μg/ml of this hormone preparation, no significant level of lens regeneration was observed (Figure 9). No lens regeneration was stimulated at all at 300 and 3 μg/ml, and

Figure 9. Lens regeneration stages achieved by dorsal irises after 20 days of culture with or without porcine adrenocorticotropin preparation ACTH-6002; 300 µg/ml ACTH-6002 = 26 I.U./ml. No significant differences were noticed between control and hormone-treated irises. See Figure 6 for further explanation of the histograms.


ACTH-6002
CONTROLS
stages
1
2
3
4
5
6
7
8
irises
18
12
6
300 µg/ml
15
10
3
2
15
10
2
2
1
30 µg/ml
15
10
3
2
16
14
2
3 µg/ml
15
10
3
2

only 20% (3 of 15) of the irises in cultures supplemented with 30 μg/ml of this hormone preparation had lens regenerates. One of these was an early vesicle at stage 4. This compares to 13% (2 of 15) regeneration occurring up to stage 3 in the corresponding controls.

3. EFFECT OF BOVINE PROLACTIN PREPARATION PRL-B4

3.1 ORGAN CULTURE

3.1.1 Regeneration Stages. Bovine prolactin preparation PRL-B4 did not significantly affect lens formation at concentrations ranging from 1600 to 23 μg/ml (Figure 10). No lens regenerates were present in any of these cultures, although some thickened dorsal iris margins of stage 3 occurred in the controls.

4. EFFECT OF BOVINE SOMATOTROPIN PREPARATION GH-B18

4.1 ORGAN CULTURE

4.1.1 Regeneration Stages. Three concentrations of bovine somatotropin preparation GH-B18 were used, ranging from 3000 to 30 μg/ml. Although some relatively advanced stages, up to a lens vesicle of stage 5, were obtained at the highest concentration, they were not very frequent and therefore not statistically significant (Figure 11). With 3000 μg/ml, 18% (4 of 17) lens regeneration was stimulated as compared to 13% (2 of 15) in controls. With 300 μg/ml, lenses started to regenerate in 7% (1 of 15) of the irises as compared to none in controls, and with 30 μg/ml of GH-B18, 6% (1 of 18) lens

Figure 10. Lens regeneration stages achieved by dorsal irises after 20 days of culture with or without bovine prolactin preparation PRL-B4; 1600 μg/ml PR1-B4 = 29 I.U./ml. The differences between controls and hormone-treated groups were not significant. See Figure 6 for further explanation of the histograms.

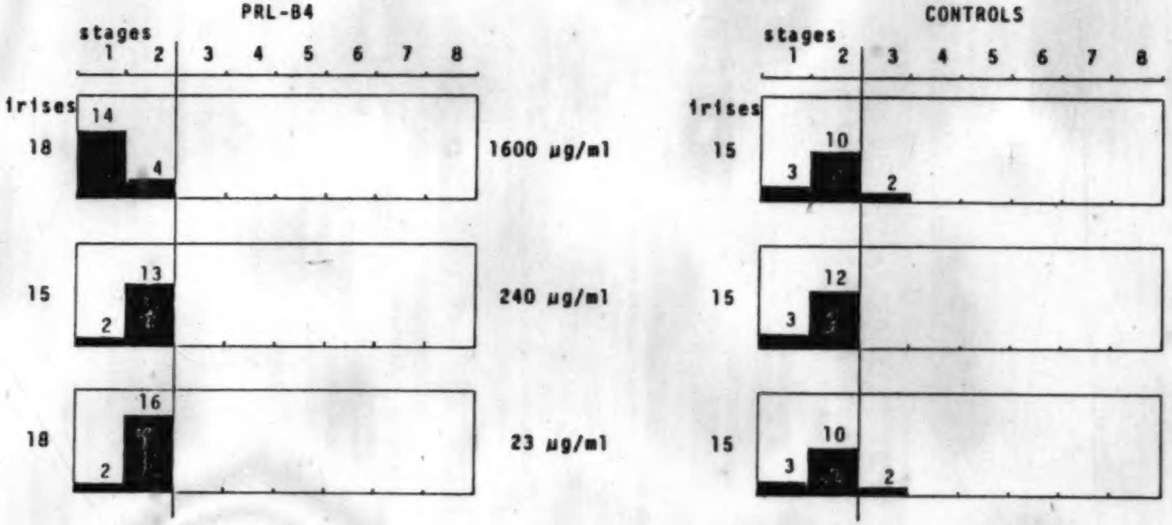
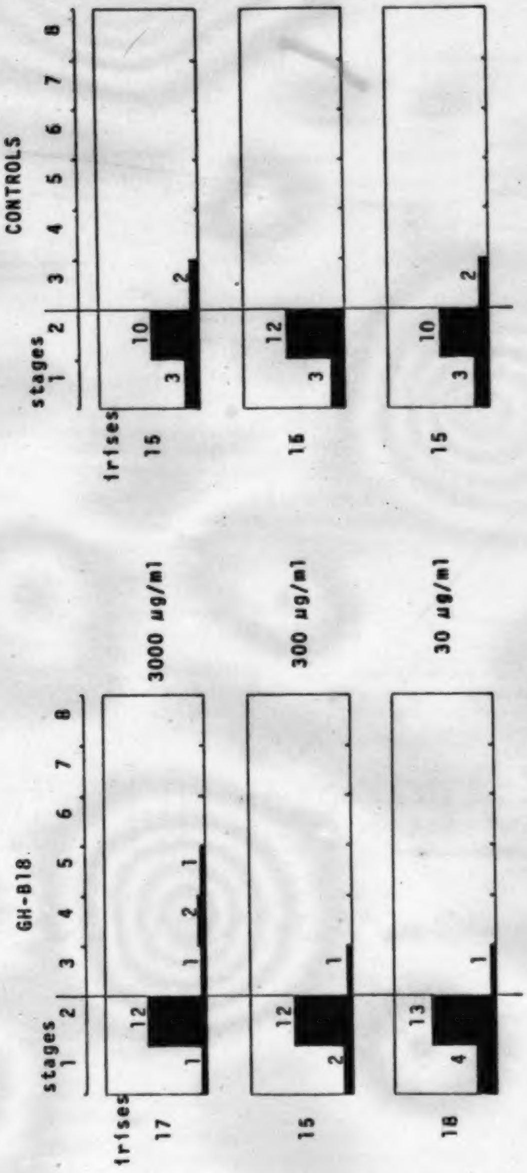

PRL-B4
CONTROLS
stages
1
2
3
4
5
6
7
8
irises
18
14
4
1600 µg/ml
15
13
2
240 µg/ml
18
16
2
23 µg/ml
15
3
10
2
15
3
12
15
3
10
2

Figure 11. Lens regeneration stages achieved by dorsal irises after 20 days of culture with or without bovine somatotropin preparation GH-B18; 3000 μg/ml GH-B18 = 2.0 I.U./ml. No significant differences (Chi square) were found between GH-B18-treated and control cultures, although the highest concentration of GH-B18 gave rise to a few lens vesicles. The differences between control and hormone-treated groups were not significant. See Figure 6 for further explanation of the histograms.


CONTROLS
GH-818
stages
1
2
3
4
5
6
7
8
irises
3000 µg/ml
300 µg/ml
30 µg/ml
17
12
1
1
2
1
15
2
12
1
18
4
13
1
15
3
10
2
16
3
12
15
3
10
2

regeneration occurred as compared to 13% (2 of 15) in the controls.

5. EFFECT OF BOVINE LUTROPIN PREPARATION LH-B10

5.1 ORGAN CULTURE

5.1.1 Regeneration Stages. The bovine lutropin preparation LH-B10 was used at concentrations ranging from 3000 to 30 μg/ml. No stimulatory effect on lens regeneration could be observed (Figure 12). Although 13% (2 of 15) of the control irises had thickened dorsal iris margins of stage 3, none of the irises cultured in the presence of 3000 μg/ml of LH-B10 started lens regeneration. With a concentration of 270 μg/ml the frequency of lens regeneration was only 6% (1 of 15) as compared to no regeneration in the controls, and with LH-B10 at 30 μg/ml, 17% (2 of 12) of the irises compared to 13% (2 of 15) in controls underwent early regeneration. Only the very early regeneration stage 3 was found in LH-B10 supplemented cultures.

6. EFFECT OF BOVINE FOLLITROPIN PREPARATION FSH-B1

6.1 ORGAN CULTURE

6.1.1 Regeneration Stages. Bovine follitropin FSH-B1 was used at concentrations between 2700 and 30 μg/ml. Only the highest concentration significantly stimulated lens regeneration in 29% (4 of 14) of the irises, while no lens regeneration was evident in controls (Figure 13). The frequencies of 22% (4 of 18) lens regenerates at 300 μg/ml and 10% (2 of

Figure 12. Lens regeneration stages achieved by dorsal irises after 20 days of culture with or without the bovine lutropin preparation LH-B10; 3000 μg/ml LH-B10 = 2.2 NIH-U/ml. The differences between treatments were not significant. See Figure 6 for further explanation of the histograms.

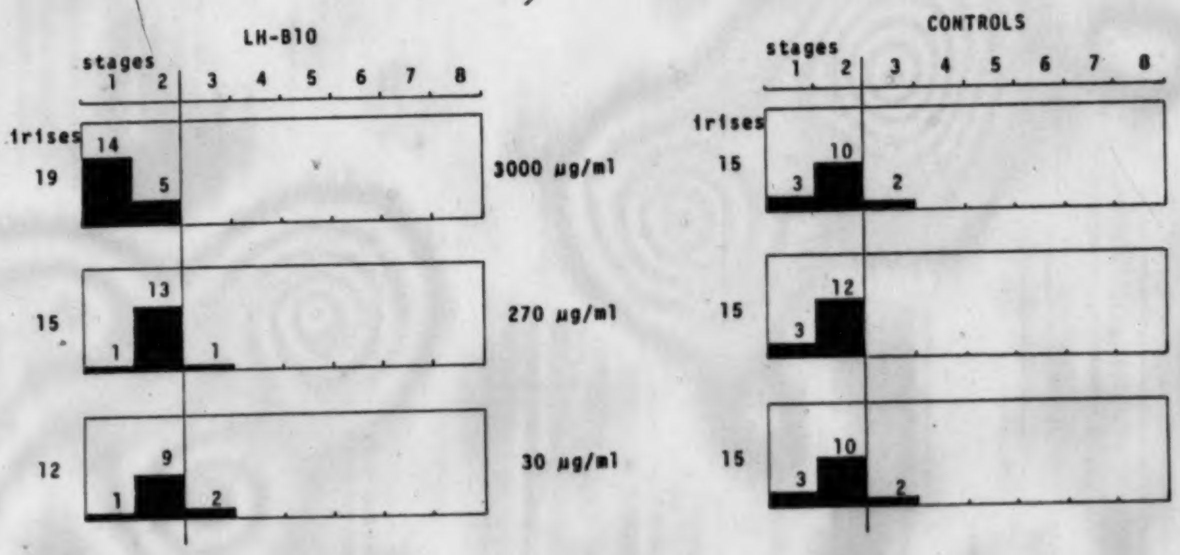
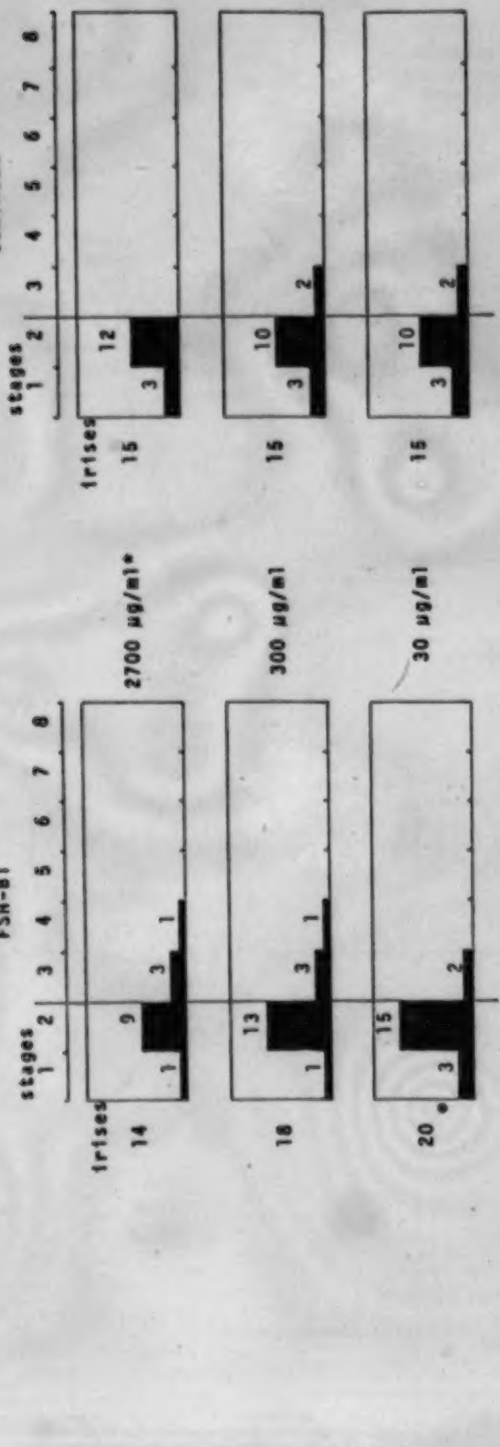

LH-B10
CONTROLS
stages
1
2
3
4
5
6
7
8
irises
19
14
5
3000 µg/ml
15
3
10
2
15
1
13
1
270 µg/ml
15
3
12
12
1
9
2
30 µg/ml
15
3
10
2

Figure 13. Lens regeneration stages achieved by dorsal irises after 20 days of culture with or without the bovine follitropin preparation FSH-B1; 2700 μg/ml FSH-B1 = 1.3 NIH-U/ml. Except for cultures supplemented with the highest concentration of FSH-B1, no significant differences (Chi square) have been found. *$P < 0.05$, no asterisks - not significant. See Figure 6 for further explanation of the histograms.


CONTROLS
stages
1
2
3
4
5
6
7
8
irises
15
3
12
15
3
10
2
15
3
10
2
FSH-B1
stages
1
2
3
4
5
6
7
8
irises
14
1
9
3
1
2700 µg/ml*
18
1
13
3
1
300 µg/ml
20
*
3
15
2
30 µg/ml

20) at 30 μg/ml were not significantly different from those of 13% (2 of 15) in both of their controls. Only early stages of lens regeneration were obtained with FSH-B1, the most advanced being a lens vesicle of stage 4.

7. EFFECT OF BOVINE THYROTROPIN PREPARATION TSH-B8

7.1 ORGAN CULTURE

7.1.1 Regeneration Stages. Dorsal irises were cultured in the presence of the bovine thyrotropin preparation TSH-B8 at concentrations ranging from 3000 μg/ml to 0.3 μg/ml (Figures 14, 20, 21). Lens regenerates at stages 3 and higher were observed in 27% (5 of 18) of the irises in cultures with 3000 μg/ml TSH-B8, and 69% (11 of 16) were regeneration-positive in cultures supplemented with 300 μg/ml of this hormone preparation, as compared to none(0 of 15) and 13% (2 of 15) in their respective controls. Lower concentrations of TSH-B8 tested during this study, 30 μg/ml and 3 μg/ml also significantly stimulated lens regeneration. At these concentrations, in two replicate experiments 43% (6 of 14, Figure 14) and 65% (11 of 17, Figure 20), or 50% (6 of 12, Figure 21) and 58% (7 of 12, Figure 21) respectively, of the irises developed lens regenerates, compared to 13% (2 of 15, Figure 14) and 17% (3 of 17, Figure 20), or 11% (2 of 19, Figure 21) and 6% (1 of 17, Figure 21) respectively, in their controls. At the lowest concentration of TSH-B8 tested, 0.3 μg/ml, only 22% (4 of 18, Figure 21) of the irises started regenerating a lens compared

Figure 14. Lens regeneration stages of dorsal irises after 20 days of culture with or without bovine thyrotropin preparation TSH-B8; 3000 μg/ml TSH-B8 = 10.5 I.U./ml. The frequency of specific lens regeneration stages (stages 3 and higher) is significantly higher in TSH-B8-treated cultures than in controls. The significance levels (Chi square) are: $^{***}P < 0.005$, $^{*}P < 0.05$, and $^{O}P < 0.10$ (almost significant). See Figure 6 for further explanation of the histograms.

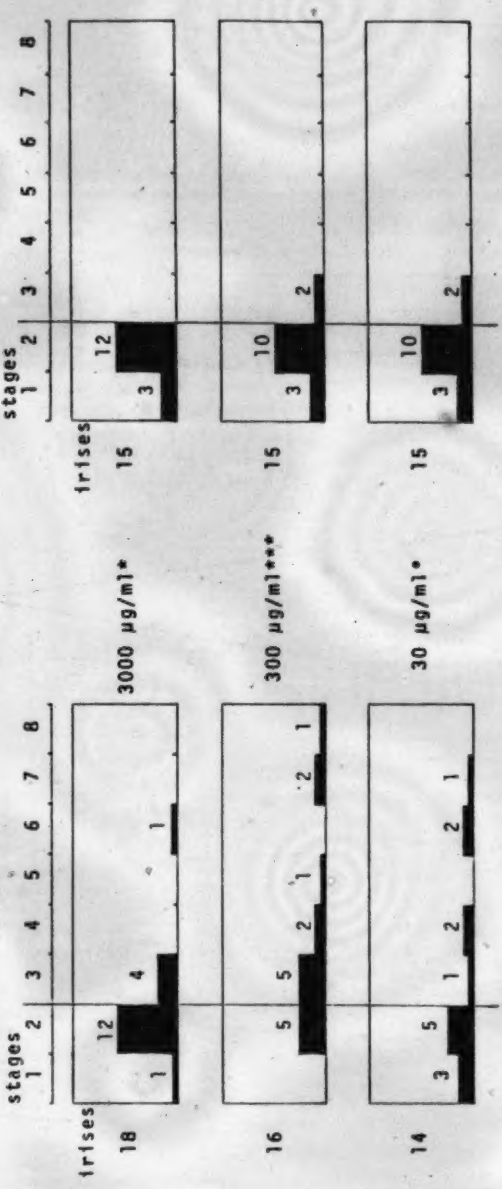
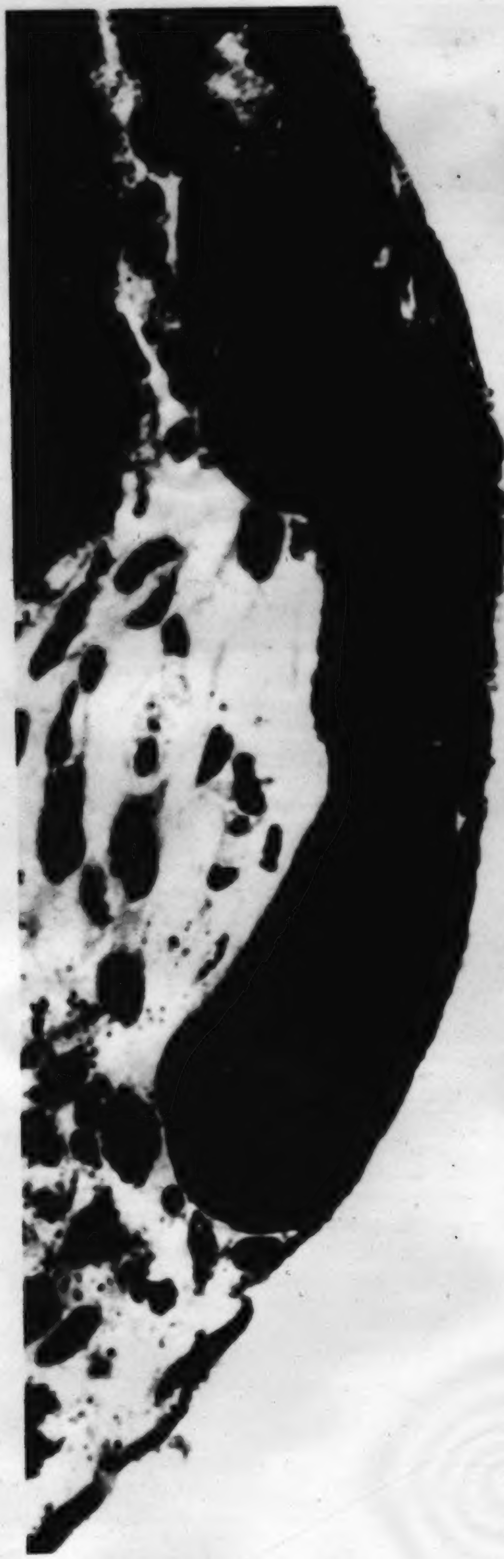
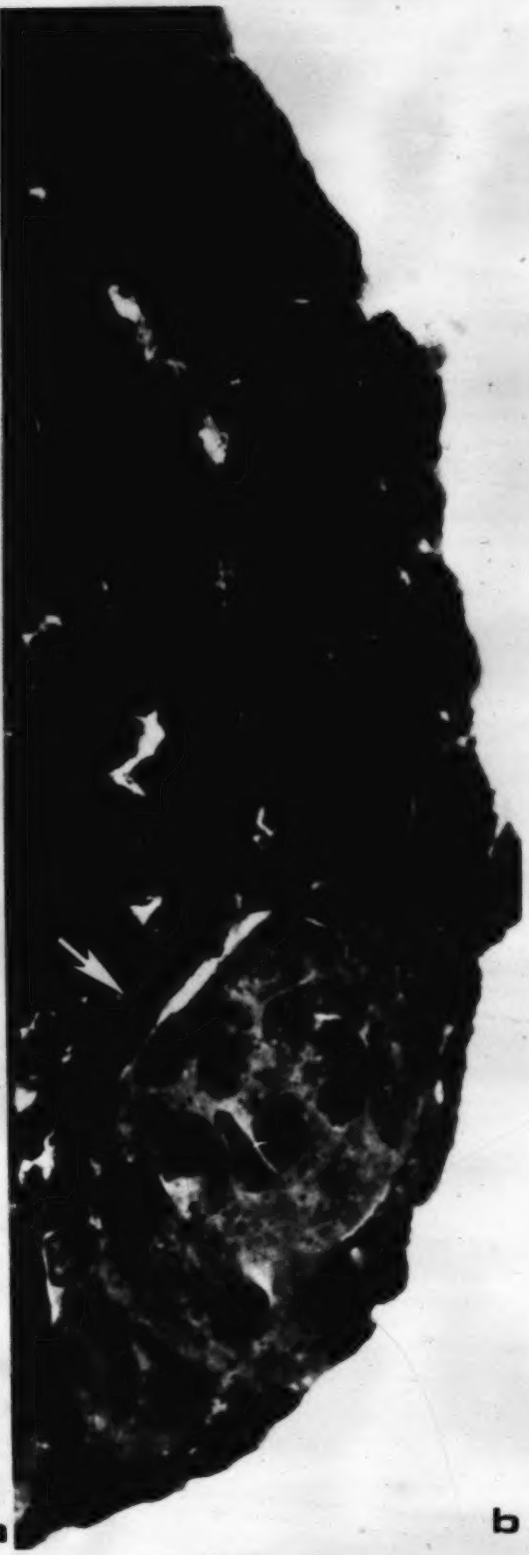

CONTROLS
stages
1
2
3
4
5
6
7
8
irises
15
15
15
3
12
3
10
2
3
10
2
TSH-BB
3000 µg/ml*
300 µg/ml**
30 µg/ml*
18
16
14
1
12
4
1
5
5
2
1
2
1
3
5
1
2
2
1

Figure 15. Lens regenerates which developed on dorsal irises after 20 days of culture with the bovine thyrotropin preparation TSH-B8. (a) Stage 1 of lens regeneration in a control culture. X 795. (b) Stage 7 obtained with 300 μg/ml of TSH-B8. Notice the lens epithelium-like region at the inside of the fiber core (arrow). X 672. Stained with hematoxylin-eosin.

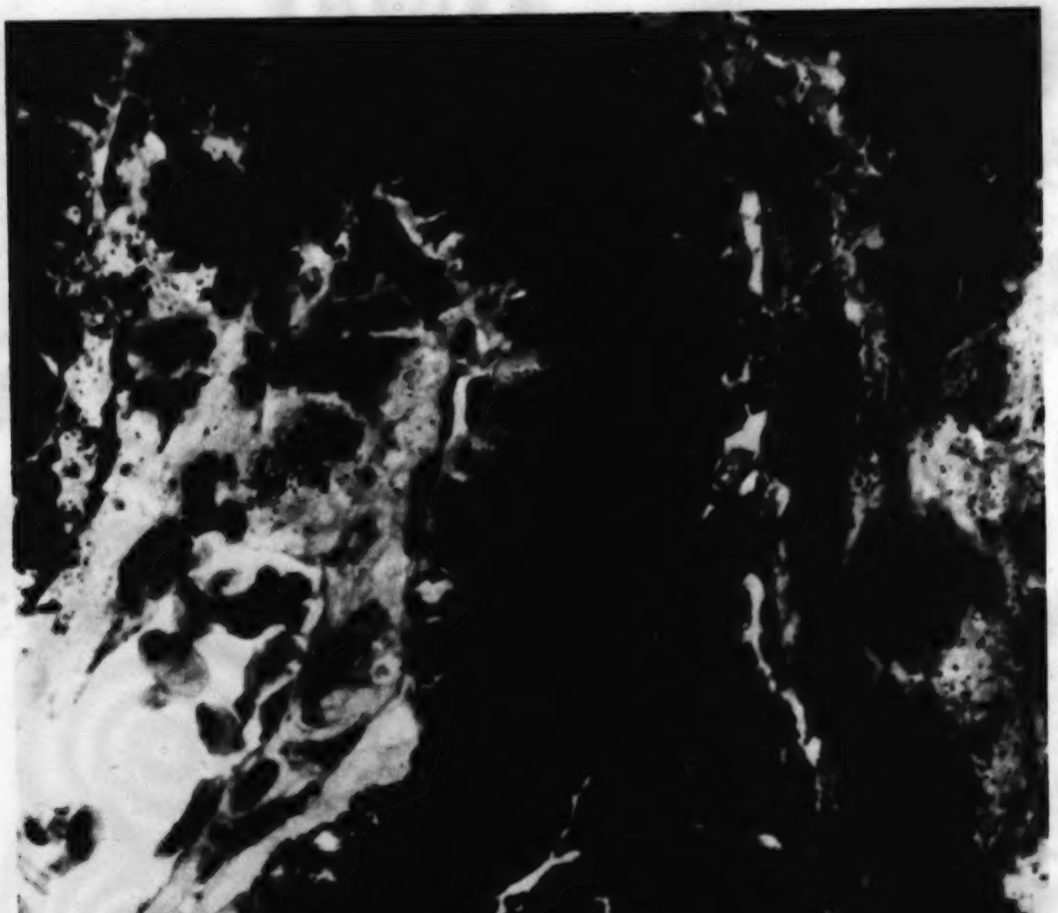


a



b

Figure 16. Lens regenerate of stage 6 which developed on the dorsal iris after 20 days of culture in the presence of 3000 μg/ml of TSH-B8. Notice the prevalence of the lens epithelium-like region. X 723. Stained with hematoxylin-eosin.



to 11% (2 of 19) in their controls. This faint effect was no longer significant.

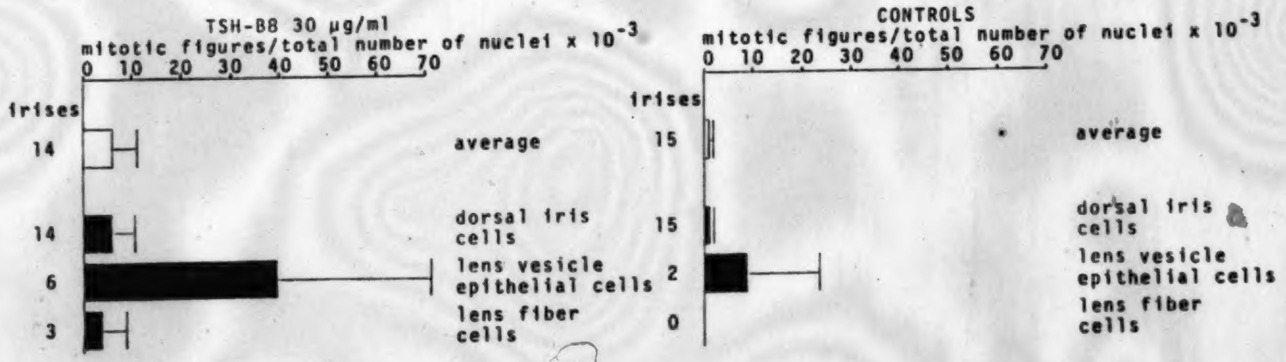
In addition to the significantly higher frequency of lens regenerates on dorsal irises cultured in the presence of TSH-B8, lens differentiation was also further advanced. At the highest concentration of TSH-B8, 3000 μg/ml, lens regeneration stage 6, characterized by the beginning of lens fiber development, was reached (Figure 16), while only the iris depigmentation stage 2 was attained in the corresponding controls. Similarly, at a TSH-B8 concentration of 300 μg/ml (Figures 14, 15) regenerates up to stage 8 characterized by the beginning of secondary lens fiber cell elongation had formed, as opposed to thickened iris margins at only stage 3 in their controls. Thirty μg/ml of TSH-B8 stimulated lens regenerates to develop up to stage 7 characterized by a lens with a primary lens fiber hillock in the first experiment, and up to stage 9 consisting of a lens with a large fiber core and with secondary fiber cells in the second experiment (Figure 22). These results contrast with those of the controls, in which only regenerates of stage 3 were obtained in the first experiment and one lens vesicle of stage 4 in the second experiment. Lens regeneration did not proceed as far at the lower concentration of TSH-B8, 3 μg/ml. In the two replicate experiments performed, stages 6 and 4, as opposed to 4 and 3 in their respective controls, were reached (Figure 21). At the lowest concentration tested, 0.3 μg/ml (Figure 21), no lens regeneration stages higher than thickened depigmented iris margins of stage 3 developed, although one early lens vesicle of stage 4 was present in the controls.

7.1.2 Mitotic Activity. No mitotic activity is detectable in the undisturbed dorsal iris (Yamada and Roesel, 1971). This process reaches maximal values between 7 to 25 days after lentectomy (Eisenberg and Yamada, 1966), from the period of depigmentation of dorsal iris cells up to the time of the detachment of the young lens from the iris margin. I determined whether the average mitotic activity in irises stimulated with 30 µg/ml of TSH-B8 would similarly be increased, by estimating the mitotic index of irises after 20 days of culture (Figure 17):

$$\text{mitotic index} = \frac{\text{nuclei in mitosis}}{\text{total nuclei per tissue}}$$

Mitotic figures (late prophase to early telophase) were counted in histological sections of dorsal irises. Care was taken to avoid counting the same mitotic figures repeatedly when they were present in several consecutive sections of the iris. The number of mitotic figures was slightly underestimated, because early prophases and late telophases were difficult to detect. The total number of nuclei present in the dorsal irises could only be estimated, because the nuclei of heavily pigmented cells were often concealed. Therefore, nuclei were counted in a depigmented area. The ratio of the counted area to the

Figure 17. Mitotic activity in dorsal irises after 20 days of culture with or without 30 μg/ml of TSH-B8 (same irises as in Figure 14). The estimated average mitotic index (mitotic figures/total number of nuclei) is significantly (Wilcoxon's rank test) elevated in TSH-B8-supplemented cultures when compared to the controls. $^{**}P < 0.01$. In irises which had lens regenerates, mitotic indices could be estimated separately for lens fiber cells, lens vesicle epithelium-like cells and dorsal iris epithelial cells. The mitotic activity pattern, with the highest activity in lens vesicle epithelium-like cells, and the lowest activity in lens fiber cells, corresponds well to the activity pattern described for lens regenerates *in vivo*.


TSH-B8 30 µg/ml
mitotic figures/total number of nuclei x 10^-3
0
10
20
30
40
50
60
70
irises
14
average
14
dorsal iris cells
6
lens vesicle epithelial cells
3
lens fiber cells
CONTROLS
mitotic figures/total number of nuclei x 10^-3
0
10
20
30
40
50
60
70
irises
15
average
15
dorsal iris cells
2
lens vesicle epithelial cells
0
lens fiber cells

total area covered by the section was estimated, and the counted number of nuclei was multiplied by that ratio. The apparent number of nuclei per iris was obtained by adding up the number of nuclei estimated for each section. The same nuclei could be followed through 2 to 4 subsequent sections. Therefore, the apparent amount of nuclei per dorsal iris was divided by 3 in order to arrive at an estimate of the total number of nuclei present in each dorsal iris. Since, on the average, nuclei in control cultures were smaller than nuclei in experimental cultures, the number of nuclei in the control cultures was probably underestimated, whereas the number of nuclei in experimental cultures was probably overestimated. This inadequacy may have given rise to an underestimation of the mitotic index in experimental cultures and an overestimation in control cultures. Therefore, the difference of the mitotic indices between control and experimental cultures is expected to be greater than indicated here.

Although there was a large variability among TSH-B8-treated irises, the average mitotic index was 5.5 times higher than that of the controls, and this difference was found to be significant, when Wilcoxon's rank test was applied. The average mitotic index mainly reflects the mitotic index of iris epithelial cells not involved in the formation of the lens, since these cells constitute a large portion of the explant. In irises with lens regenerates it was possible to determine mitotic indices separately for lens fiber cells, lens vesicle epithelium-like cells, and dorsal iris epithelial cells. The distribution of mitotic activity in the lens regenerates was similar to that found in the *in vivo* situation, with the lens fiber region having the lowest mitotic activity and the remaining lens cells attaining the highest mitotic activity (Figure 17).

7.1.3 Presence of γ-Crystallin. Gamma-crystallin is the last lens specific protein to appear during lens regeneration in the newt (Takata et al., 1966), and in young lenses *in situ* it is confined to lens fiber cells. I have tested for presence of γ-crystallin in lens regenerates as an additional criterion for lens differentiation, using an immunofluorescence technique. That antibodies directed against frog γ-crystallin also react specifically with newt γ-crystallin has been documented (Nöthiger et al., 1971). I have tested the monospecificity of the anti-frog γ-crystallin antiserum by immunoelectrophoresis (Figure 18). Although three different crystallins were present in the soluble fraction of frog lens homogenates, the antiserum formed only one precipitation arch corresponding to γ-crystallin. In addition, when sections of newt eyes with 30 day lens regenerates of stage 11 were stained, only the lens fiber core was fluorescent (Figure 19), but not the lens epithelium or any other part of the eye. The relative abundance of γ-crystallin in lens regenerates was roughly classified in three groups: (+) only a few weakly

Figure 18. Specificity of anti-frog γ-crystallin antiserum for frog γ-crystallin. (a) Immunoelectrophoresis of frog aqueous lens extract against anti-frog γ-crystallin antiserum only yields one (γ-crystallin) precipitation arch, whereas (b) anti-frog α, β, γ-crystallin antiserum yields 3 (α, β, γ-crystallin) precipitation arches.

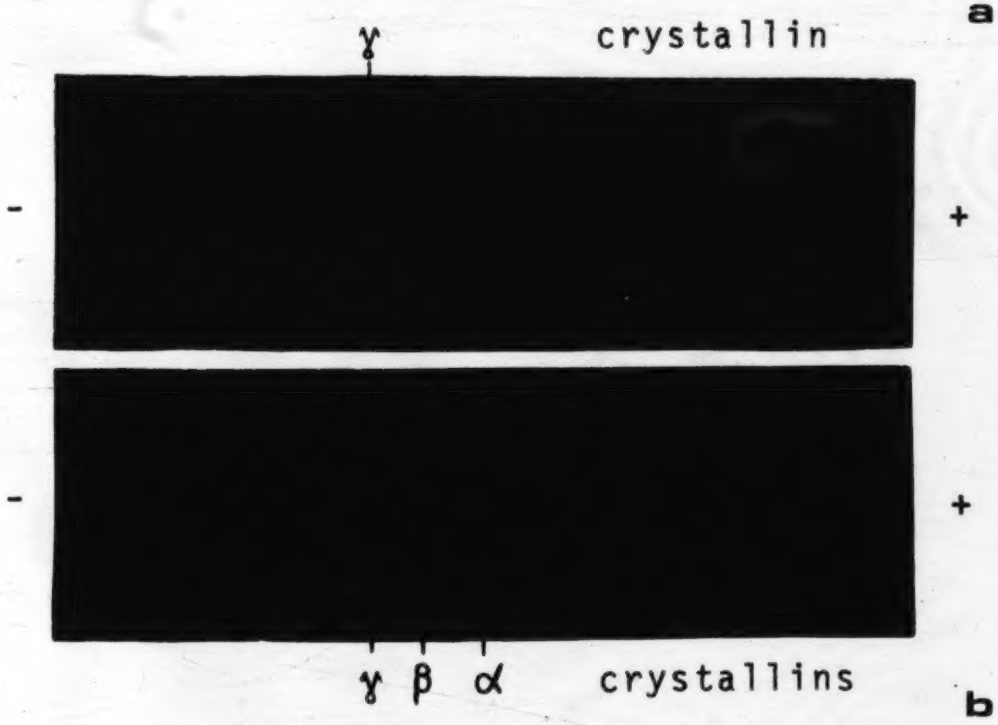

γ crystallin
a
-
+
-
+
γ β α crystallins
b

Figure 19. Specificity of anti-frog γ-crystallin antiserum for young newt lens fiber cells *in vivo*. Eye cross sections with stage 11 lenses, 30 days after lentectomy, were stained with anti-frog γ-crystallin antiserum or unimmunized serum. (a) Transmission light micrograph, (b) corresponding immunofluorescent light micrograph with unimmunized serum. No fluorescence is detectable. (c) Transmission light micrograph with anti-frog γ-crystallin antiserum, and (d) corresponding immunofluorescent light micrograph. Note that the lens epithelium remains unstained (arrow). (a,b,c,d) X 30.8.

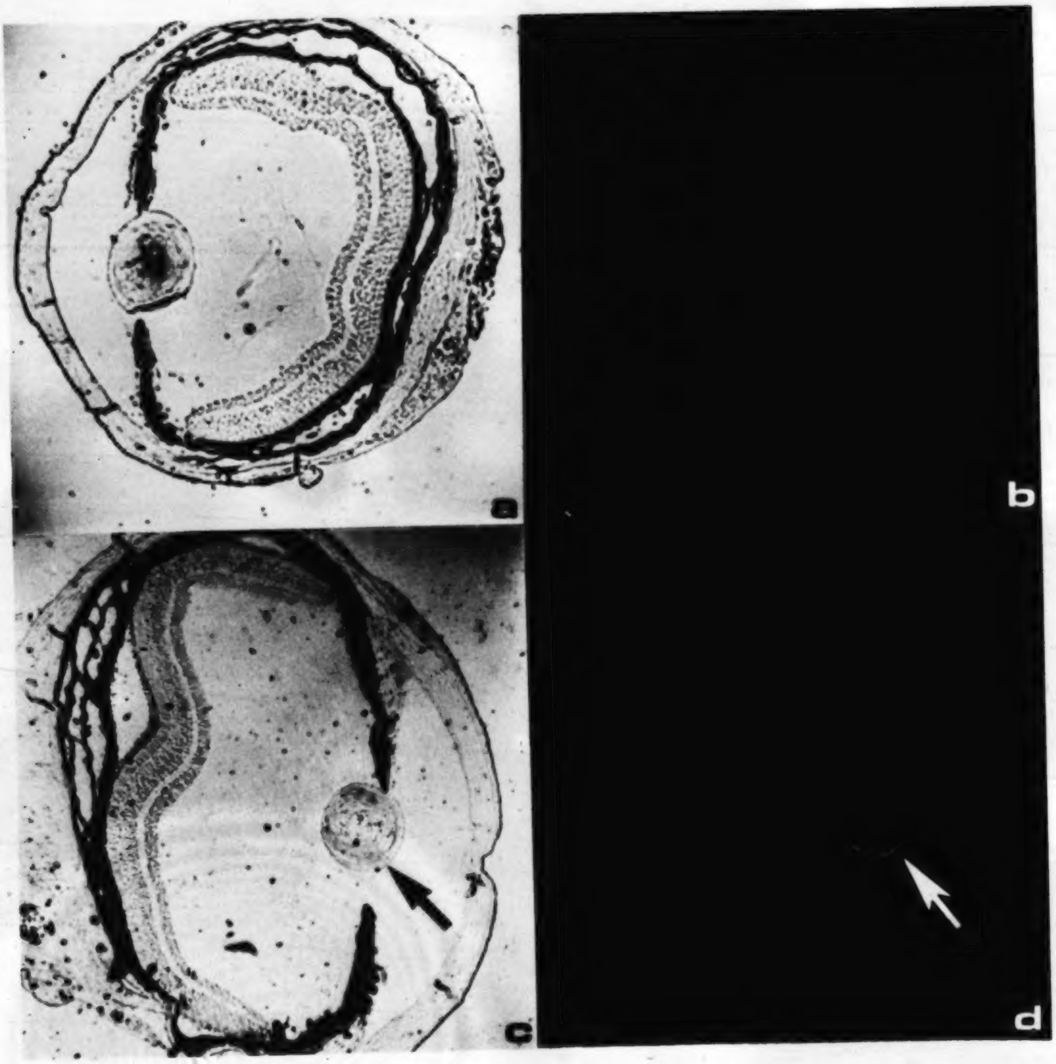

a
b
c
d

Figure 20. Gamma-crystallin accumulation in lens regenerates of dorsal irises after 20 days of culture with or without bovine thyrotropin preparation TSH-B8. The significance level (Chi square) for the difference in frequency of specific lens regeneration stages (stages 3 and higher) for TSH-B8-treated cultures as compared to controls is: **P < 0.01. See Figure 6 for further explanation of the histograms, and Figure 7 for explanation of the + and - symbols.

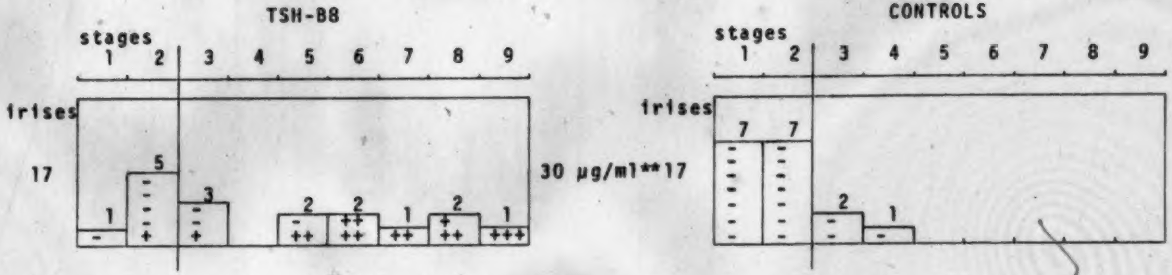

TSH-B8
stages
1
2
3
4
5
6
7
8
9
irises
17
1
-
5
-
-
-
-
+
3
-
-
+
2
-
++
2
++
++
1
++
2
+
++
1
+++
30 µg/ml**17
CONTROLS
stages
1
2
3
4
5
6
7
8
9
irises
7
7
-
-
-
-
-
-
-
2
-
-
1
-

Figure 21. Lens regeneration stages of dorsal irises after 20 days of culture with or without bovine thyrotropin preparation TSH-B8; 3.0 μg/ml TSH-B8 = 0.01 I.U./ml. The TSH-B8 preparation used here had been stored dry at -20°C for almost 3 years prior to use. The frequency of specific lens regeneration (stages 3 and higher) is significantly higher in TSH-B8-treated cultures than in controls, down to a concentration of 3 μg/ml TSH-B8. The significance level is: $*P < 0.01$, no asterisks, not significant. The presence of γ-crystallin was confirmed by immunofluorescence in some TSH-B8-treated lens regenerates. See Figure 6 for further explanation of the histograms and Figure 7 for explanation of the + and - symbols.

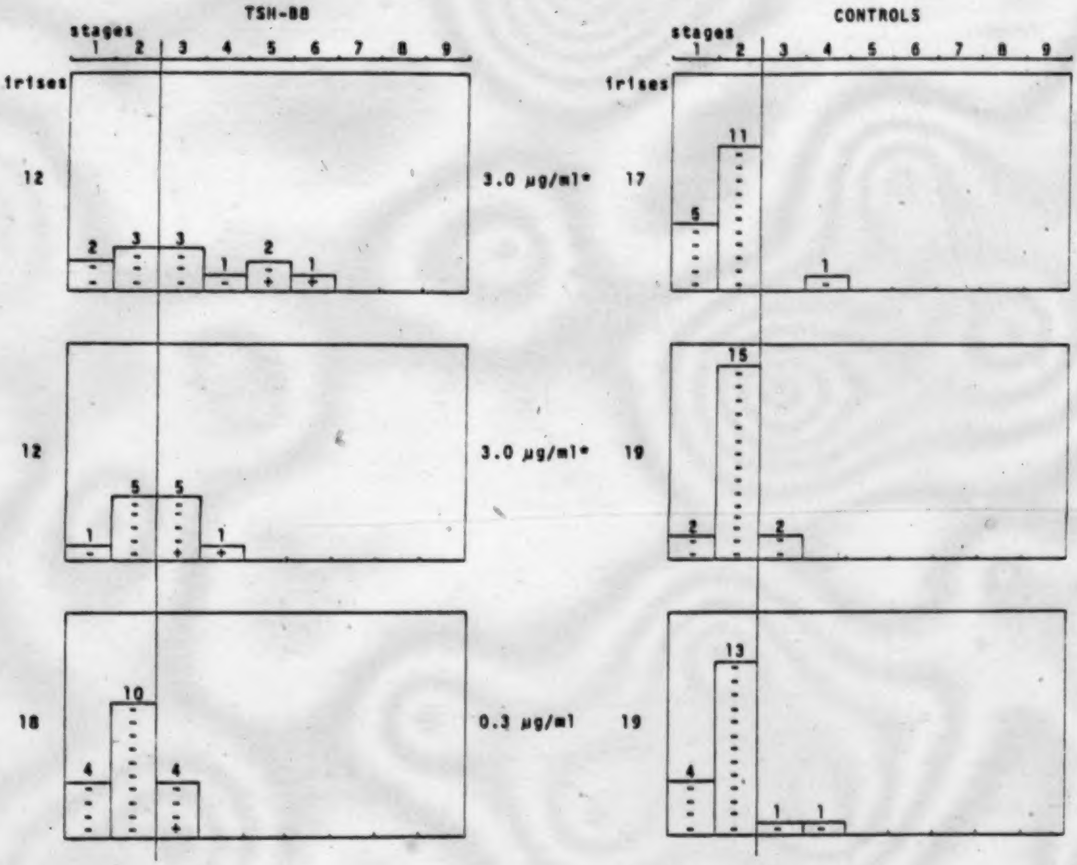

TSH-BB
CONTROLS
stages
1 2 3 4 5 6 7 8 9
irises
12
3.0 µg/ml*
17
2 3 3 1 2 1
5 11 1
12
3.0 µg/ml*
19
1 5 5 1
2 15 2
18
0.3 µg/ml
19
4 10 4
4 13 1 1

Figure 22. Gamma-crystallin accumulation in a lens regenerate of stage 9 which developed on a dorsal iris after 20 days of culture in the presence of 30 μg/ml of TSH-B8. (a) Stained transmission light micrograph, (b) corresponding immunofluorescent light micrograph stained with anti-frog γ-crystallin antiserum. (a,b) X 317. Note that the lens epithelium-like cells are not stained (arrow).

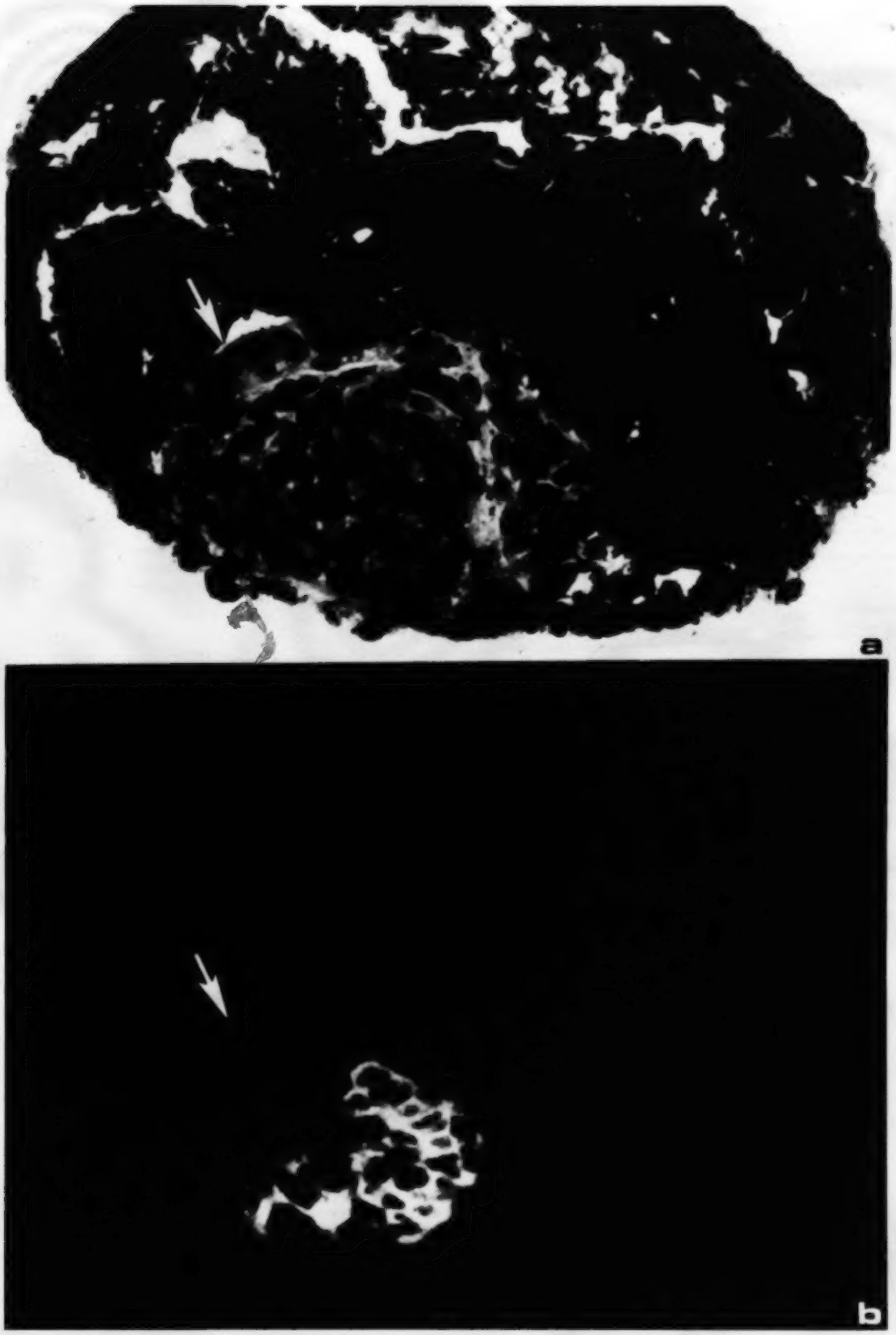

a
b

Figure 23. Gamma-crystallin accumulation in a lens regenerate of stage 6 which developed on a dorsal iris after 20 days of culture in the presence of 30 μg/ml of TSH-B8. (a) Stained transmission light micrograph, and (b) corresponding immunofluorescent light micrograph stained with anti-frog γ-crystallin antiserum. Note that the lens epithelium-like cells are not stained and tend to repigment (arrow). (a,b) X 334.

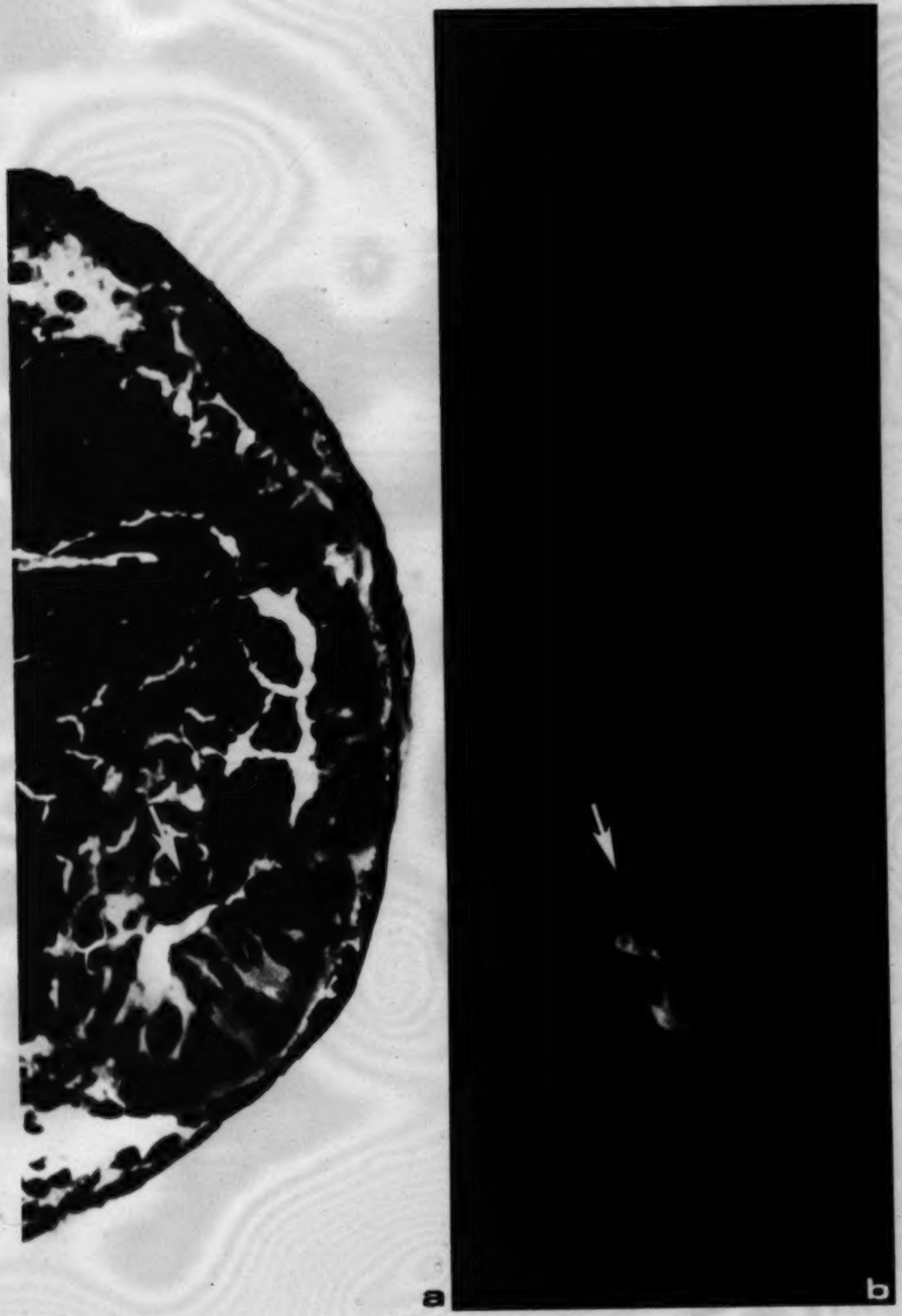

a
b

Figure 24. Gamma-crystallin accumulation in one cell of a lens regenerate of stage 3, which developed on a dorsal iris after 20 days of culture in the presence of 0.3 μg/ml of TSH-B8. (a) Stained transmission light micrograph, and (b) corresponding immunofluorescent light micrograph stained with anti-frog γ-crystallin antiserum. (a,b) X 280. Note the mitotic activity in this early lens regenerate.

a
b

fluorescent cells, (++) a cluster of 11 to 50 strongly fluorescent cells, and (+++) a large lens fiber core with over 100 strongly fluorescent cells. The presence of γ-crystallin was found to be well correlated with the morphologically estimated lens regeneration stages (Figures 20, 21, 22, 23), although a few deviations were observed. *In vivo*, regenerates up to the lens vesicle stage 4 do not contain γ-crystallin. Under the present culture conditions, however, groups of cells positive for γ-crystallin can occur in regenerates classified as stage 2 or 3, in cultures supplemented with TSH-B8 down to concentrations of 0.3 μg/ml (Figure 24).

7.2 CELL CULTURE: GENERAL CONSIDERATIONS

The behaviour of dissociated iris cells under cell culture conditions is described in this chapter.

7.2.1 Mitotic Activity. Generally, cultured iris cells in mitosis partially detach from the plastic substratum and become spherical (Yamada, 1977; Figure 26). Also, the condensed chromosomes are often visible under phase contrast optics and cell division can therefore be observed in living cells. To determine the mitotic activity attached spherical cells were counted every 5 days in one half of every dish and the number obtained was multiplied by 2 to arrive at the total number of dividing cells per dish. The occasional mitotic figures in attached flattened cells and in completely detached cells were missed and some nondividing spherical lentoid cells were unintentionally included in the counts. In addition, mitotic cells present in cell clusters could not be counted. The mitotic index was not estimated, because it would have been too difficult to count cells organized in clusters, especially in those of a pigmented nature. However, the precision of counting was good (Table 3). To estimate the error of counting, a dish was counted 5 consecutive times, always rotating it by an arbitrary angle between counting sessions, so as to obliterate memory and recognition effects. In each experiment, control and treated cultures were derived from the same suspension of dissociated cells and the number of cells per dish was comparable in all dishes of an individual experiment. The first mitotic figures were encountered 10 (Figures 36, 39, 42) to 15 days after explantation (Figures 27, 28, 29) and were often due to dividing stromal fibroblasts. During the incubation time of the cultures several poorly defined irregular peaks of mitotic activity were observed; the first one appeared after 20 to 30 days, the second after 40 to 50 days, the third after 65 to 75 days, and the fourth peak after 85 to 95 days of culture (Figures 27, 28, 29). The mitotic activity peaks are best evident in relatively pure iris epithelial cell cultures (Figure 28), possibly because there is no overlap with the different cell cycle activities of the iris stromal fibroblasts (compare Figure 29). The initial cell density in the different experimental series varied from approximately 1.4

5

Table 3. Estimation of counting error in cell cultures. The culture dish (Figure 29) was counted 5 consecutive times at day 92 of culture. The dish was rotated at an arbitrary angle between counting sessions, so as to obliterate memory and recognition effects.

counting session	lentoid bodies	iridal bodies	mitotic figures
1	68	97	202
2	69	107	218
3	64	108	208
4	63	103	224
5	64	99	226
mean and range	65.6 ± 3.4	102.8 ± 5.8	215.6 ± 13.6
percent variability between counts	10.4%	11.2%	12.6%

to 23 times. However, the initial cell density was the same in treated and control cultures of each experiment. In high density mixed cell cultures (Figure 27) only the first and third peak of mitotic activity were observed, and the third peak was vestigeal. It is possible that suppression of the peaks of mitotic activity in high density cultures can be ascribed to the shorter time needed by the cultured cells to reach saturation density. In low density cell cultures these waves of mitotic activity did not decrease as rapidly (Figures 28, 29, 36, 39, 42) as in high density cultures. In low to very low density mixed iris cell control cultures (Figures 36, 39, 42), the first peak of mitotic activity was shifted to a later date by about 15 days (compare Figures 27, 28, 29). The high and moderately low cell density cultures were performed between winter and spring, whereas the low and very low cell density cultures were conducted between spring and summer. Because of the above mentioned variation in the distribution of mitotic activity in time, controls were always performed parallel to stimulation experiments using thyrotropin TSH-B8.

7.2.2 Iridal Bodies. Under cell culture conditions, iris epithelial cells have a tendency to form clusters, whereas stromal fibroblasts grow mainly in sheets. These two cell types initially tend to sort out and iris epithelial cells aggregate in dense three-dimensional pigmented clusters, the iridal bodies (Figure 32), whereas the stromal fibroblasts remain attached and spread on the plastic substratum. However, in cultures older than about 70 days these cell types may start to mix, and clusters are often not as firm and regularly shaped as in young cultures (Figures 33, 35). Young iridal bodies are usually completely black (Figure 32), whereas in older cultures they are composed of cells at various degrees of depigmentation. Clusters composed of three-dimensional aggregates consisting of over 50% partially to totally pigmented iris epithelial cells, which had risen above the level of single attached cells or cell sheets, were classified as iridal bodies. They were counted in all cell culture dishes on an inverted phase contrast microscope (Wild M 20, or Zeiss IM 35) at 5 day intervals.

In some experiments a few pigmented clusters had formed by 5 days of culture (Figures 40, 43) or by 10 days (Figure 27), but in other experiments it took up to 25 days (Figure 28) for the first appearance of iridal bodies to occur. The clustering activity could be in part related to the mitotic activity, since clustering peaks follow mitotic peaks in some experiments (Figure 28). However, this relationship may not be direct, because iridal bodies can form 5 days before the first mitotic figures become visible (Figure 27). Also the peaks of mitotic and clustering activities do not always correlate (Figures 27, 42 and 43). The presence of stromal fibroblasts may not be necessary for the formation of iridal bodies since the latter were present in cultures of

Figure 25. Dorsal iris epithelial cells in cell culture. (a) Totally pigmented iris epithelial cell after 30 days of culture with 3 to 12 μg/ml TSH-B8 (same culture as in Figure 29). On observation this cell was found to be moving slowly. Notice the pseudopodia which are sent out in various directions. X 390. (b) Partially depigmented iris epithelial cell after 32 days of culture with 3 to 12 μg/ml TSH-B8 (same culture as in Figures 36 to 38). This cell has spread and attached to the plastic surface and did not move during the time of observation. Notice the shed melanosomes in the culture medium. X 340. (c,d) Lens fiber cell containing γ-crystallin in its cytosol after 100 days of culture with 3 to 12 μg/ml TSH-B8 (same culture as in Figure 29). (c) Transmission light micrograph, and (d) corresponding fluorescent light micrograph stained with anti-frog γ-crystallin antiserum. This cell was observed to move at a visible speed towards the lower edge of the picture. (c,d) X 360.

a
d

Figure 26. Dorsal iris epithelial cell in cell culture. This totally depigmented cell was photographed after 30 days of culture with 3 to 12 μg/ml of TSH-B8 (same culture as in Figure 29). It is in late anaphase of the mitotic cell cycle, and is only loosely attached to the substratum during this process. Dorsal iris epithelial cells of *Notophthalmus viridescens* are diploid and have 21 chromosomes *in vivo*, but *in vitro* they may become aneuploid (Reese et al., 1976). X 409.

Figure 27. High density mixed iris cell culture. Iris cell suspensions consisting of epithelial cells, stromal fibroblasts, and iridophores from 11 dorsal irises per dish were cultured for 110 days. In each dish 89 x 10^3 cells were innoculated; about 53 x 10^3 of them attached and spread (60%). Two dishes were supplemented with TSH-B8 varying in concentration from 3 to 12 μg/ml (solid lines), and the remaining dish served as control (dashed line). As criteria for changes in the differentiative program lentoid bodies, iridal bodies and mitotic figures were counted at intervals of 5 days. Each line and symbol represents one individual culture dish.

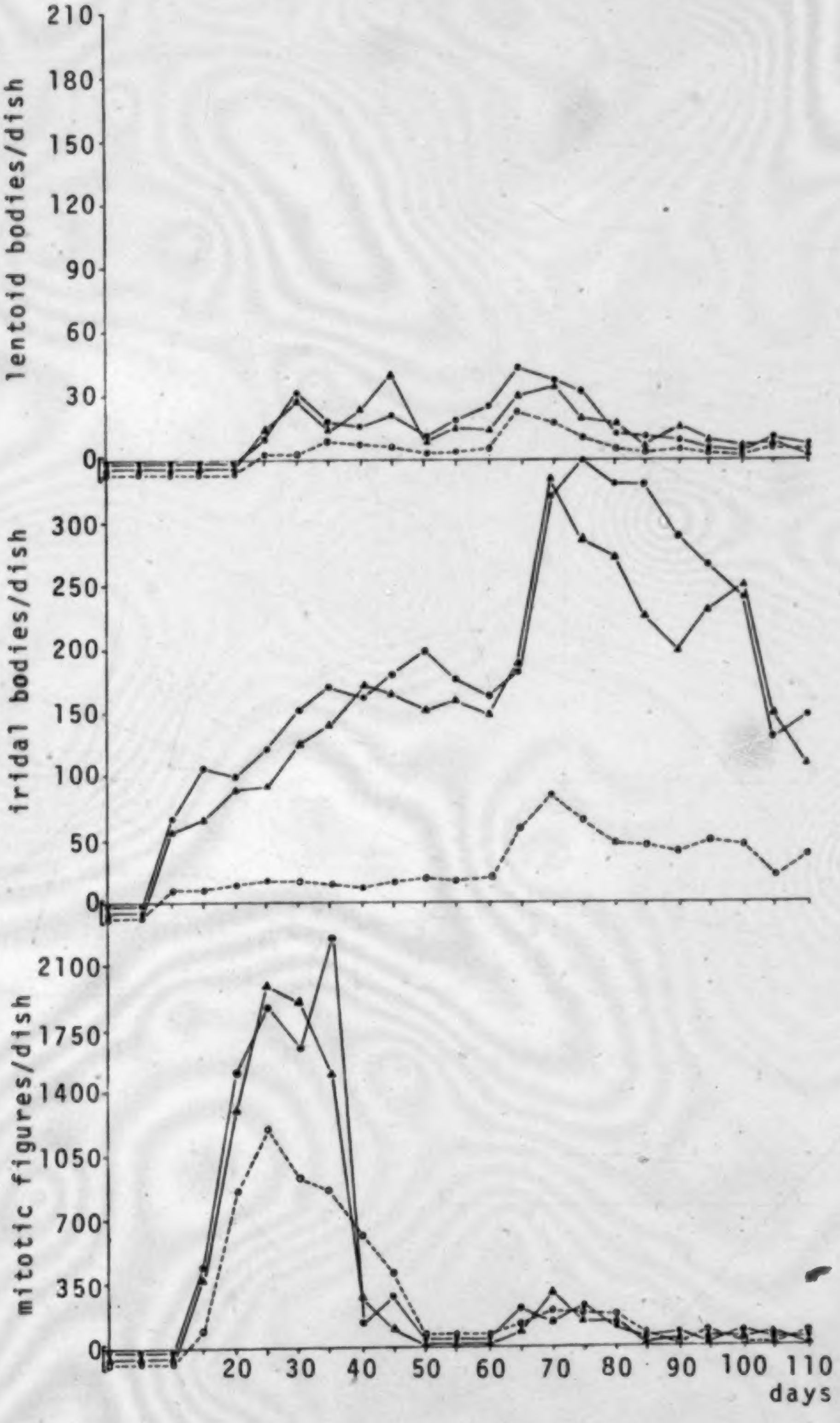

lentoid bodies/dish
210
180
150
120
90
60
30
0
iridal bodies/dish
300
250
200
150
100
50
0
mitotic figures/dish
2100
1750
1400
1050
700
350
0
20
30
40
50
60
70
80
90
100
110
days

Figure 28. Low density pure iris epithelial cell culture. Iris epithelial cells from 10 dorsal irises per dish were cultured for 110 days. About 40 x 10^3 cells were innoculated in each dish, and about 12 x 10^3 of them attached and spread (30%). Two dishes were supplemented with a varying concentration of TSH-B8, 3 to 12 μg/ml (solid lines), the remaining two dishes served as controls (dashed lines). Lentoid bodies, iridal bodies, and mitotic figures were counted in each dish at 5 day intervals. Each line and symbol represents one individual culture dish.

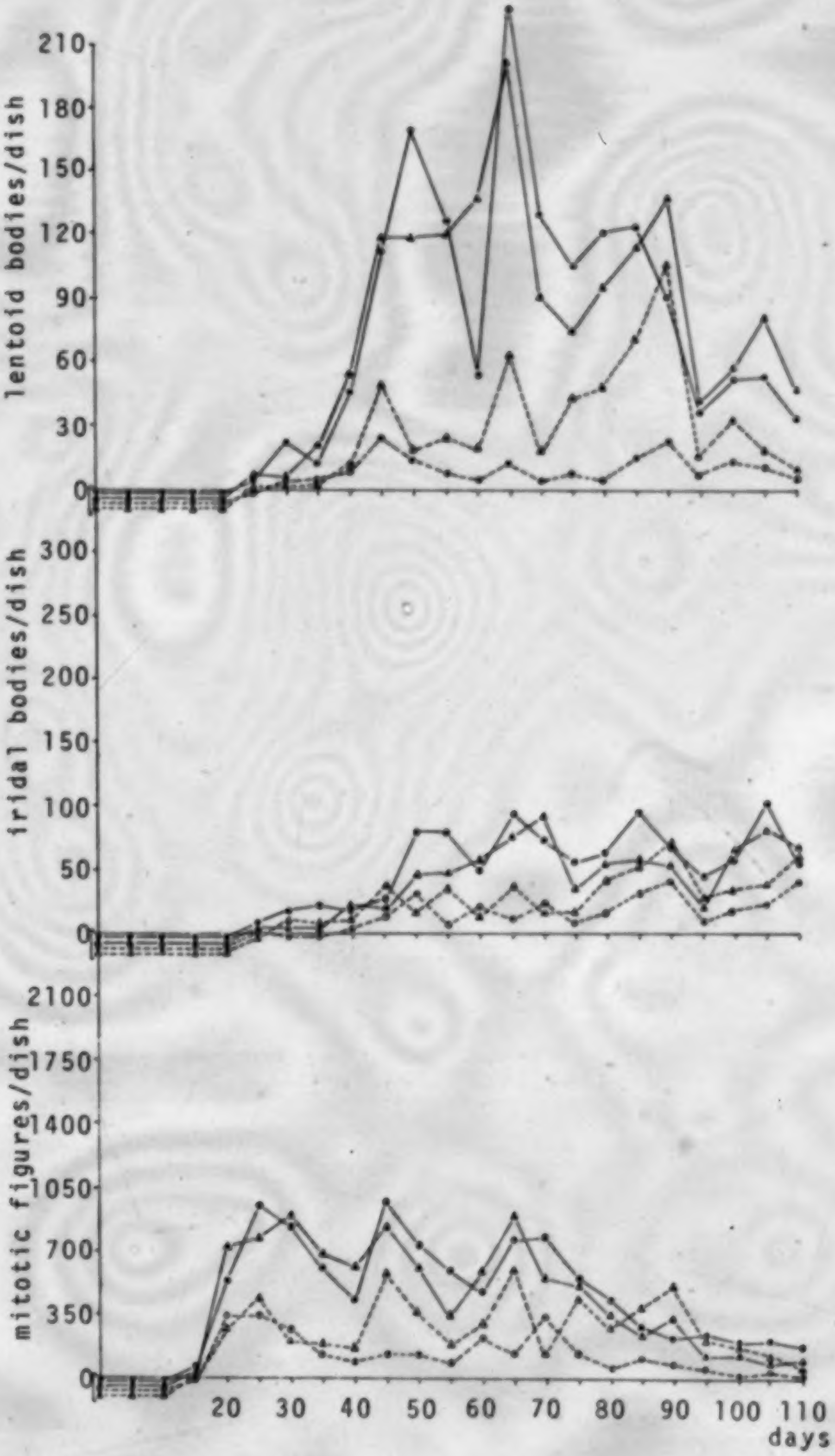

lentoid bodies/dish
210
180
150
120
90
60
30
0
iridal bodies/dish
300
250
200
150
100
50
0
mitotic figures/dish
2100
1750
1400
1050
700
350
0
20
30
40
50
60
70
80
90
100
110
days

Figure 29. Low density mixed iris cell culture. An iris cell suspension consisting of iris epithelial cells, stromal fibroblasts, and iridophores from 5 dorsal irises per dish was cultured for 100 days. About 30 x 10^3 cells were innoculated in each dish and about 15 x 10^3 of them attached and spread (50%). Four dishes were supplemented with a varying concentration of TSH-B8, 3 to 12 μg/ml (solid lines), the remaining four dishes served as controls (dashed lines). Lentoid bodies, iridal bodies, and mitotic figures were counted in each dish at 5 day intervals. Every 25 days, the best experimental and the best control culture dish were sacrificed for detection of lens specific proteins (see Figure 35). Each line and symbol represents one culture dish.

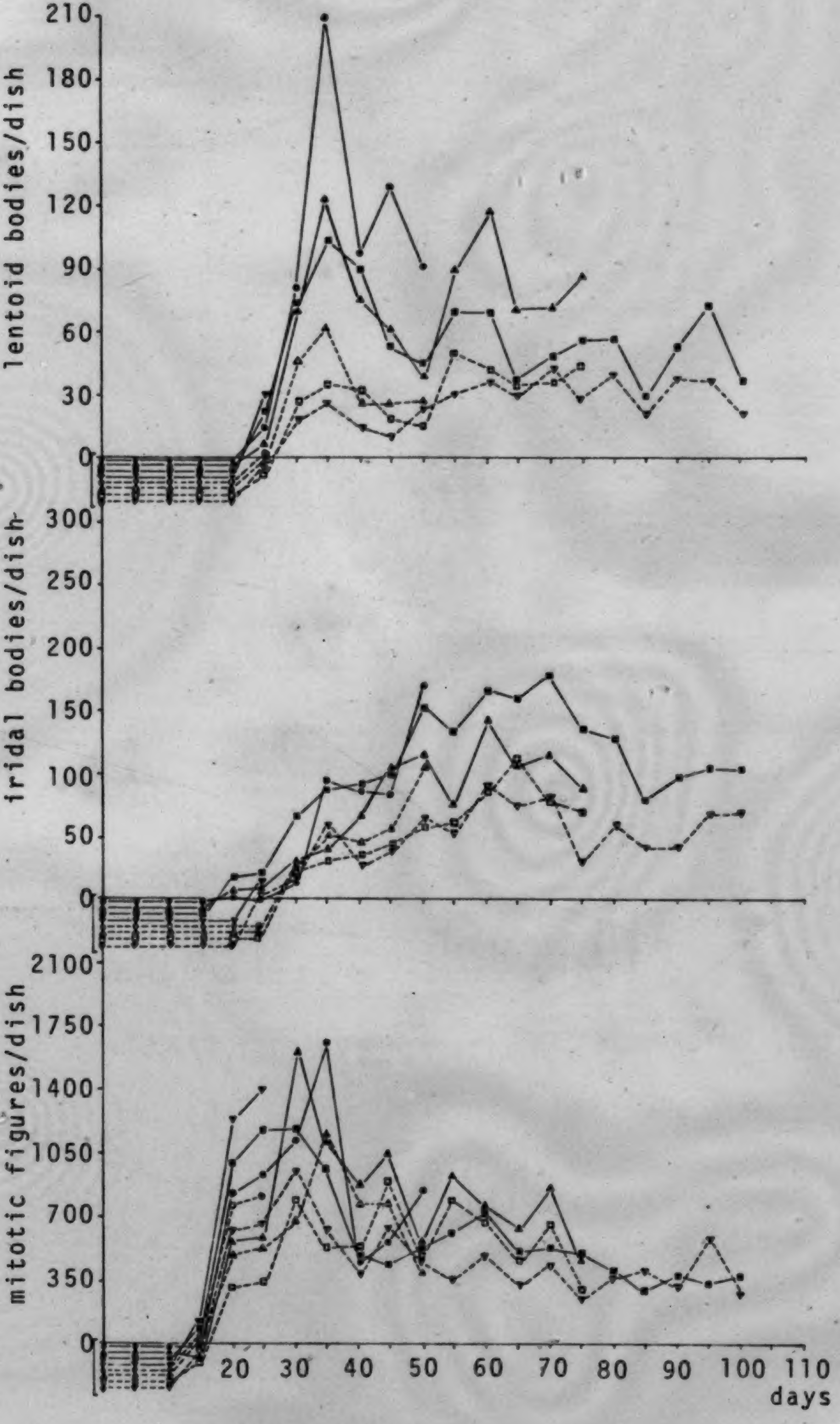

lentoid bodies/dish
210
180
150
120
90
60
30
0
iridal bodies/dish
300
250
200
150
100
50
0
mitotic figures/dish
2100
1750
1400
1050
700
350
0
20
30
40
50
60
70
80
90
100
110
days

Figure 30. Low density mixed iris cell culture 8 days after explantation (same culture as in Figure 29). (a) Supplemented with 3 to 12 μg/ml TSH-B8, (b) control culture. At this time of culture, no differences between experimental and control cultures were apparent, except that the still totally pigmented dorsal iris epithelial cells often had a closer association with one another in TSH-B8-containing cultures (a). Attachment was not complete yet, and spherical non-attached black iris epithelial cells as well as small transparent stromal fibroblasts are visible. Depigmentation has started, as is apparent from the abundance of shed melanosomes in the culture medium. (a) X 255, (b) X 250.

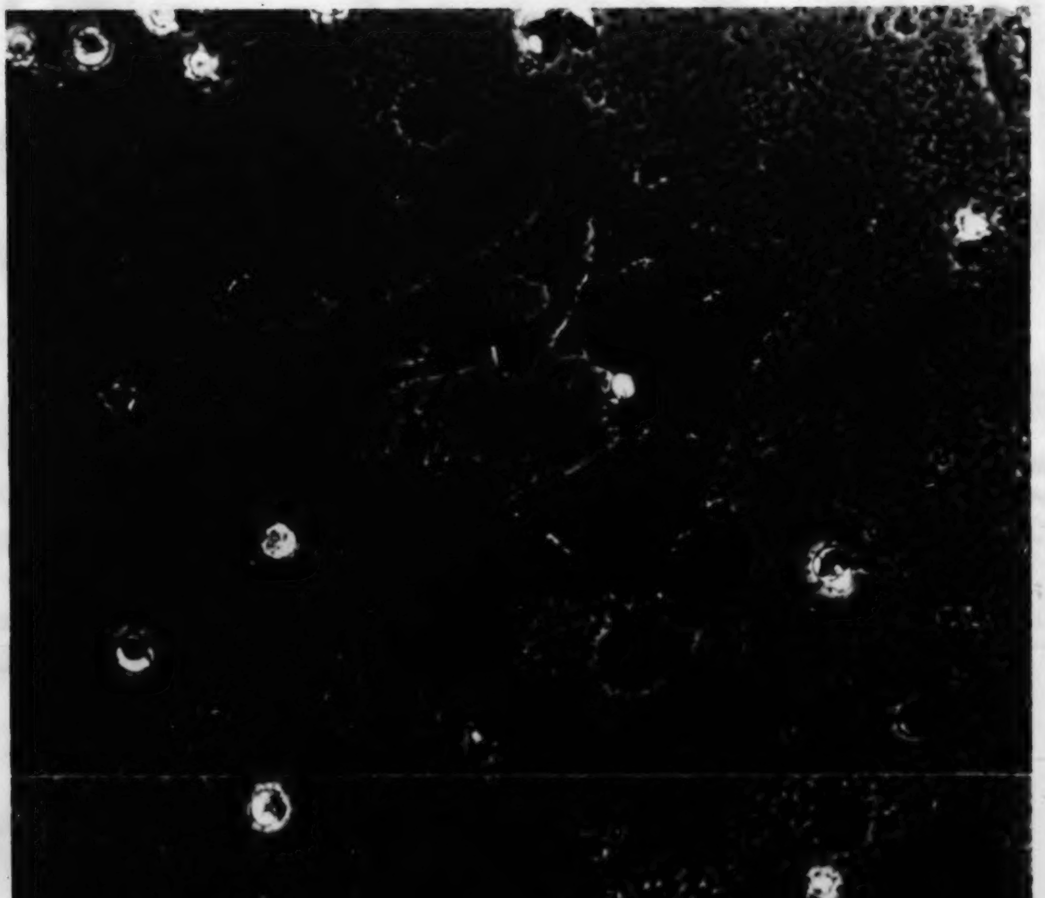

b

Figure 31. Lentoid bodies (arrow) in a low density mixed iris cell culture 30 days after explantation (same cultures as in Figure 29). (a) Supplemented with 3 to 12 μg/ml TSH-B8, and (b) control culture. Although the frequency of lentoid bodies was higher in TSH-B8-treated cultures, there was no difference in their organization or size when compared to controls. (a) X246, (b) X 253.

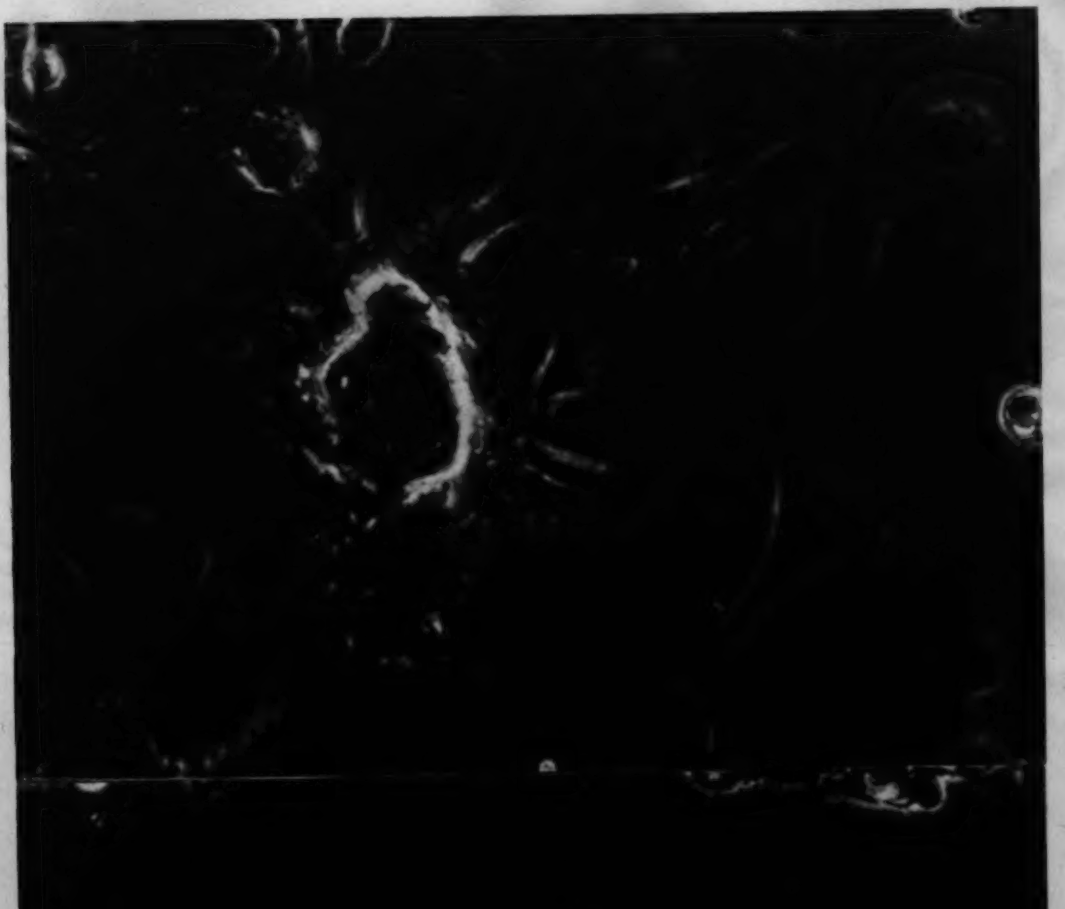


Figure 32. Pigmented iridal bodies in a low density mixed iris cell culture 30 days after explantation (same cultures as in Figure 29). (a) Supplemented with 3 to 12 μg/ml TSH-B8, and (b) control culture. Although the frequency of iridal bodies is higher in TSH-B8-treated cultures there is no difference in their morphology or size when compared to controls. Iridal bodies tend to occupy the crowded center of the culture dish, whereas lentoid bodies tend to be located further towards the periphery. (a) X 258, (b) X 250.

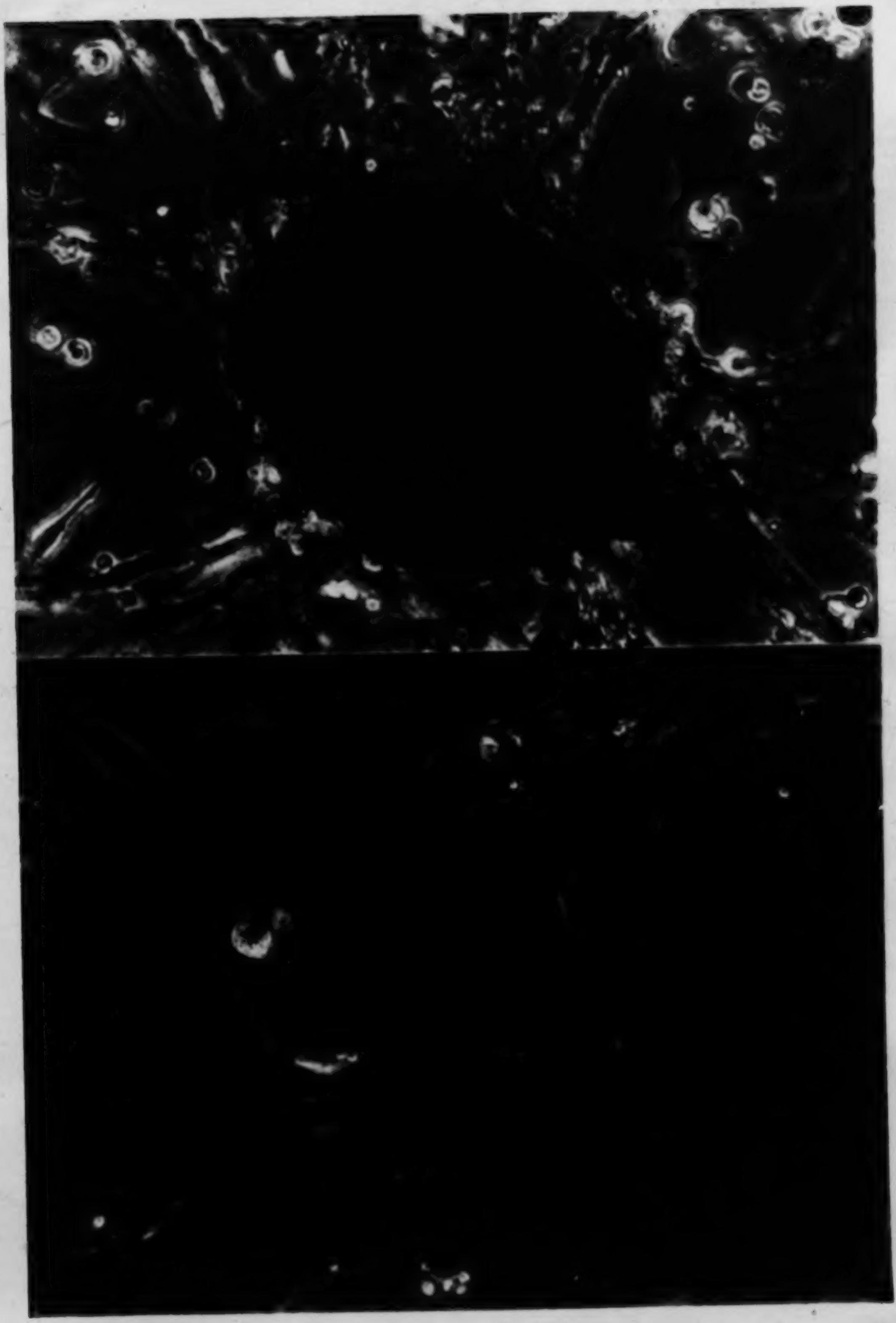
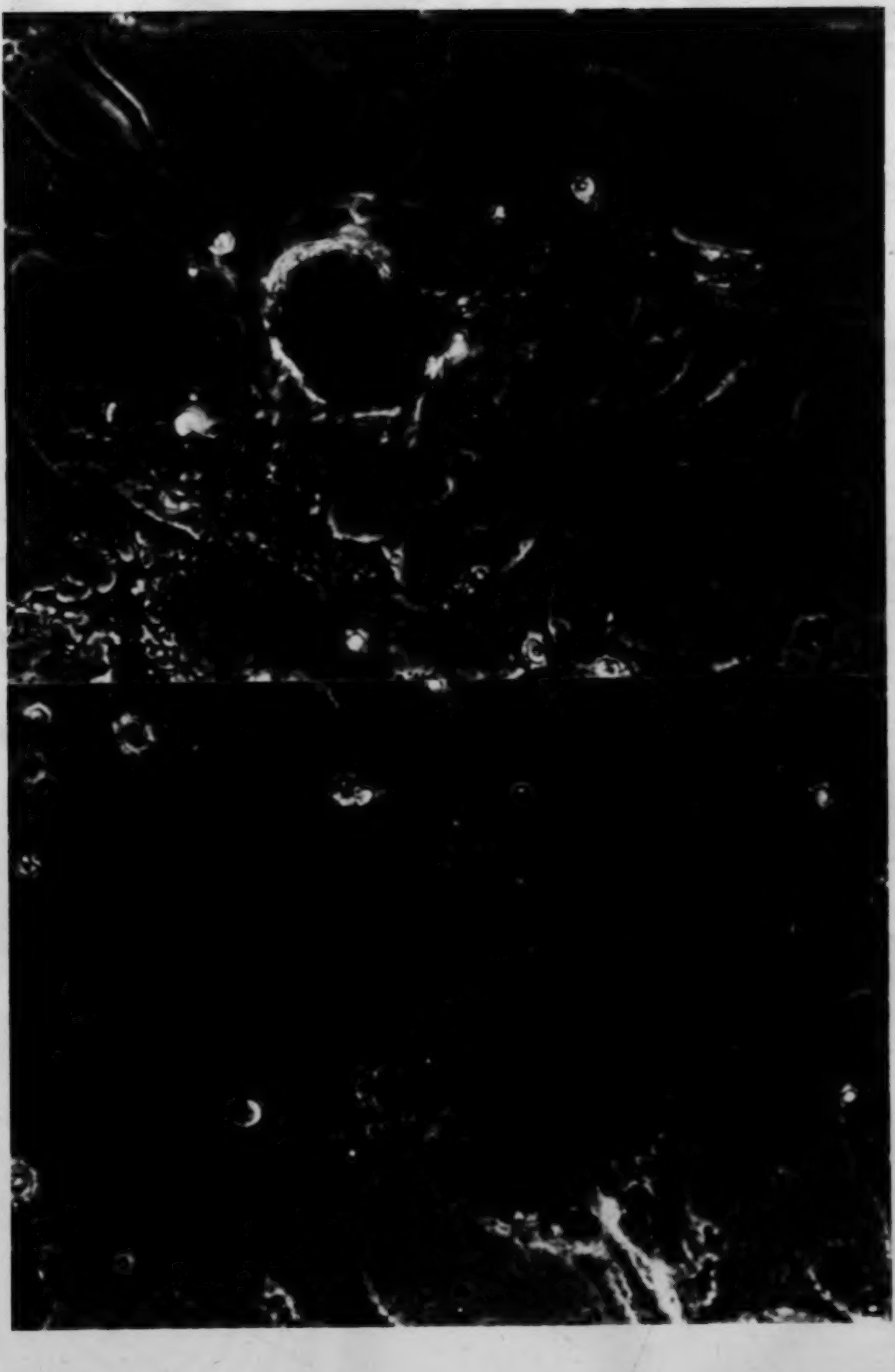


Figure 33. Low density mixed iris cell culture 30 days after explantation (same culture as in Figure 29). (a) Partially depigmented three-dimensional iridal body which formed in the presence of 3 to 12 μg/ml TSH-B8, and (b) sheet-like two-dimensional growth of mixed dorsal iris cells in a control culture. (a) X 261, (b) X 263.



relatively pure iris epithelial cells (Figure 28). This process may, however, be slightly accelerated in the presence of fibroblasts, since iridal bodies appeared five days earlier in low density mixed iris cell cultures (Figure 29) as compared to cultures of pure iris epithelial cells (Figure 28).

7.2.3 Lentoid Bodies. Highly refractile, more or less transparent clusters composed of over 50% totally depigmented iris epithelial-like and lentoid-like cells were classified as lentoid bodies (Figure 31). Apparently, lentoid bodies can be formed in different ways: (1) In younger cultures, depigmented lentoid cells migrate until they meet other lentoid cells, and aggregate to form firm, more or less spherical, and almost totally depigmented lentoid bodies. (2) In medium aged cultures, pigmented or partially depigmented iris epithelial cells aggregate to form an iridal body of spherical to elongated shape; this subsequently depigments further and transforms into a lentoid body. (3) In old cultures, senescent iridal bodies, usually with fuzzy outline, may attract freshly recruited lentoid cells to form peripheral lentoid margins. Like iridal bodies, lentoid bodies were counted at 5 day intervals. Lentoid bodies are not persistent structures due to the high mobility of all types of cultured cells. Lentoid and other cultured cells assume a spindle-shape (Figure 25 c,d) and move at a visible speed across the plastic dish. Lentoid bodies fuse, divide, or disassemble, and lentoid cells move from one body to the next. Therefore, individual cell clusters do not remain in one place and can normally not be recognized any more after 5 days.

The time of appearance of lentoid bodies seems to be independent of the presence of stromal fibroblasts or the cell density. The first lentoid bodies were noticed after 20 to 25 days (Figures 27, 28, 29, 38, 41, 44). They were more sparse in control cultures than in treated cultures. These structures appeared 10 (Figures 38, 44) to 15 days (Figures 27, 28, 29, 41) after the emergence of mitotic figures (Figures 27, 28, 29, 36, 39, 42). Thus, lentoid bodies formed about 5 to 10 days later than lens vesicles would in the newt *in vivo*. This delay is probably caused by the damage to cell surface components during dissociation. The number of lentoid bodies varies, but seems to be augmented just after mitotic peak periods (Figures 27, 28, 29, 36 and 38, 39 and 41, 42 and 44). During the culture phases characterized by a lower frequency of lentoid bodies and starting after about 40 days of culture, cataractous lentoid bodies, with cells containing large vacuoles possibly corresponding to telolysosomes (Yamada et al., 1979) were often seen. This may indicate that lentoid cells as soon as they have converted into this cell type may have difficulties surviving under our culture conditions. The difficulty of culture of adult lens fiber cells is well known (Peltz and Pezzella, 1976; Owers and Duncan, 1979). It is not possible to explain the changes in cluster frequency solely by dispersal and reaggregation of lentoid cells. It is suggested that during each peak period new cells are recruited from the population of iridal cells and are converted into lens cells. In addition, the possibility remains that some labile cells may produce melanin at one time and crystallin at another time.

If the frequencies of lentoid bodies are compared to the frequencies of iridal bodies, one can see, in general, that the proportion of lentoid bodies is higher in low density cultures (Figures 28, 29, 37 and 38, 40 and 41, 43 and 44) than in high density culture (Figure 27). This may be related to the longer lasting logarithmic growth and mitotic activity phases in low density cultures, which may increase the frequency of recruitment of convertible cells. In a dish, lentoid bodies tend to occupy more peripheral regions than iridal bodies. The center of the dishes is usually the most crowded area; in this region the mitotic activity is lower than in the peripheral regions.

7.2.4 Presence of Crystallins. Single γ-crystallin-containing cells and cell clusters positive for γ-crystallin were counted separately in dishes which had been sacrificed at 25 day intervals of culture. Dishes which did not contain γ-crystallin detectable by immunofluorescence, were subsequently exposed to anti-frog α, β, γ-crystallin antibodies to test for presence of α- and/or β-crystallins.

Generally, it may be stated that cultures which contained more lentoid bodies at the time of fixation also had more γ-crystallin-positive clusters, although γ-crystallin was present in iridal bodies as well. In one experimental series of low density mixed iris cells, cultures were harvested every 25 days (Figure 34). In the remaining experiments cultures were only screened for lens proteins at the end of the experiment after 60 (Figure 45) or 110 days (Figure 34). After 25 days (Figure 34) no γ-crystallin could be detected. When dishes were stained with anti-whole-crystallin antiserum, however, the presence of other crystallins could be demonstrated (Figure 35). Since β-crystallins are the first lens specific proteins that appear during embryonic lens development in the newt (McDevitt and Brahma, 1979, 1981), whereas α-crystallins appear last, it may well be that these first crystallins in our cell cultures were β-crystallins. After 50 days of culture all lentoid bodies and a few single cells contained γ-crystallin (Figures 34, 35). The number of cell clusters positive for γ-crystallin decreased after 75 and 100 days (Figures 34, 35), although an increasing number of iridal bodies became positive for this protein. Single γ-crystallin containing cells increased in number in the late culture period, and reached maximal frequency after 75 days in control cultures. At 100 days of culture, most clusters had a mixture of partially pigmented cells and cells positive for γ-crystallin. The rise in single lentoid cells may reflect the cell type mixing trend

Figure 34. Presence of γ-crystallin in cell culture with or without 3 to 12 μg/ml TSH-B8; (left) low density mixed iris cell culture (same as in Figure 29), (right) low density pure iris epithelial cell culture (same as in Figure 28), and (far right) high density mixed iris cell culture (same as in Figure 27). The pure low density, and the mixed high density cultures were harvested at the end point of 110 days, and were stained with fluorescein-labelled antibody for γ-crystallin detection. Mixed low density cultures were sacrificed every 25 days up to the end point of 100 days. Always the experimental (solid line, closed symbols), and the control culture (dashed line, open symbols), with the largest number of lentoid bodies, based on the data on Figure 29, were fixed. The symbols identify individual culture dishes of Figures 27, 28, and 29. The total amount of cell clusters (top of graph) has been taken from Figures 27, 28, and 29 and is the sum of iridal and lentoid bodies. Gamma-crystallin was not limited to lentoid bodies, but also occurred in some iridal bodies (center of graph). Single γ-crystallin-positive cells were also counted (bottom of graph).

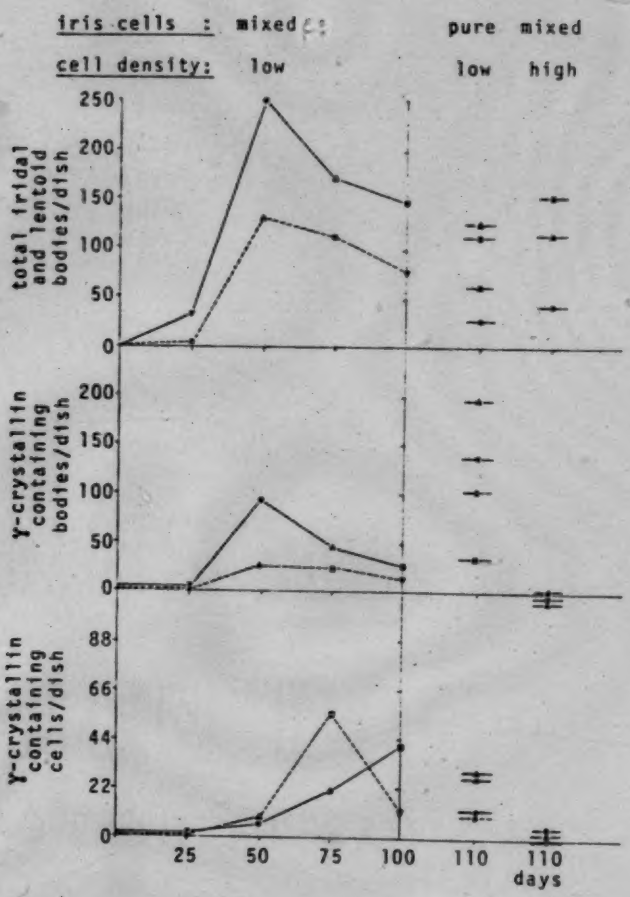
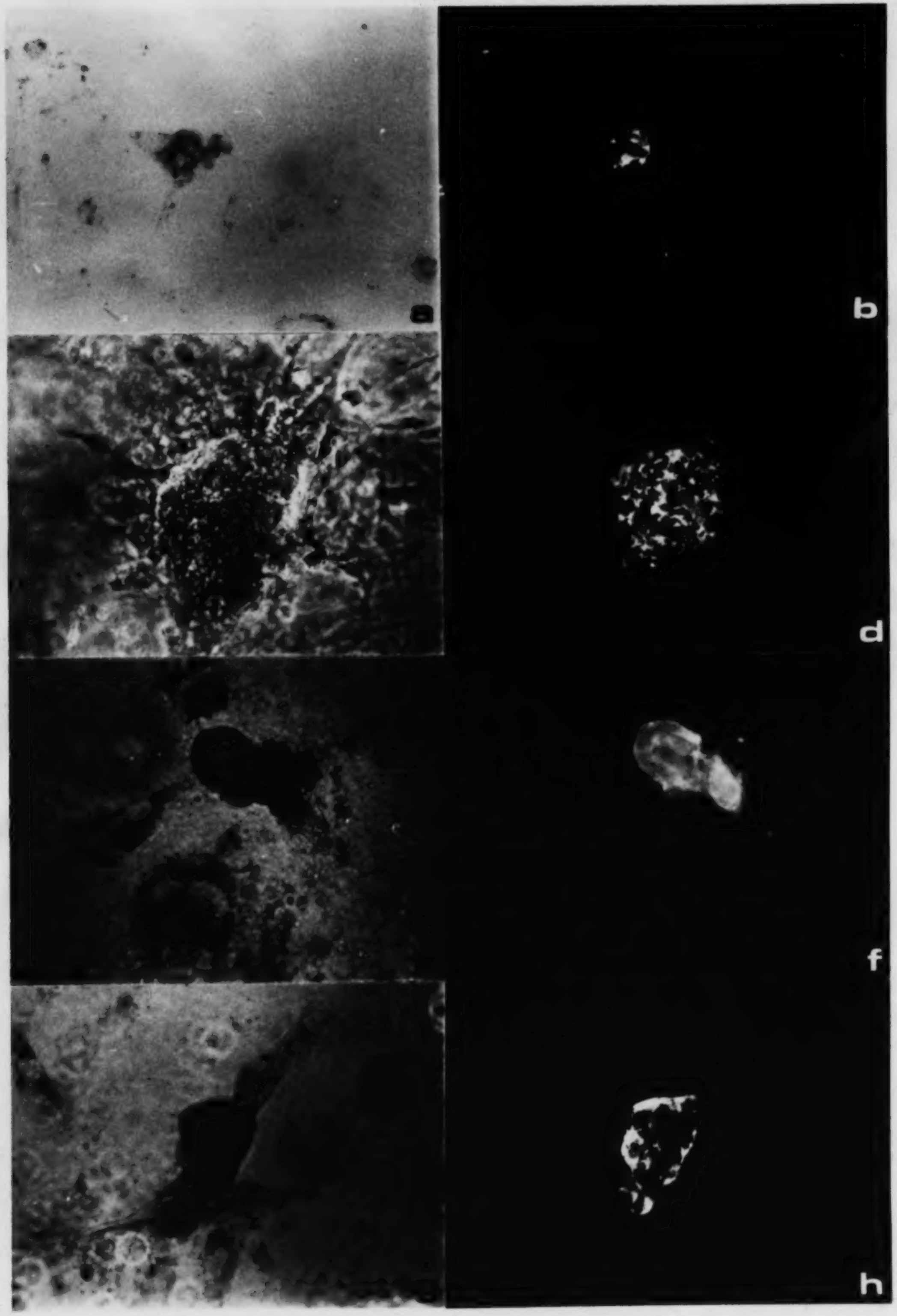

iris cells : mixed
cell density: low
pure
low
mixed
high
total iridal and lentoid bodies/dish
250
200
150
100
50
0
γ-crystallin containing bodies/dish
200
150
100
50
0
γ-crystallin containing cells/dish
88
66
44
22
0
25
50
75
100
110
110
days

Figure 35. Presence of crystallins in low density mixed iris cell culture with 3 to 12 μg/ml TSH-B8 (same as in Figures 29 and 34). (a,c,e,g) Transmission light micrographs, and (b,d,f,h) corresponding fluorescent light micrographs. After 25 days of culture, no γ-crystallin was detectable in these cultures. However, when dishes were stained again, this time with α, β, γ-crystallin antiserum (a,b), some small clusters were fluorescent, and possibly contained β-crystallins. After 50 days, large healthy looking lentoid bodies were present, packed with γ-crystallin (c,d). After 75 days, compact as well as cataractous lentoid bodies with vacuolated cells (e,f), and many iridal bodies were positive for γ-crystallin. At the end point of 100 days, only few senescent, cataractous, small or repigmenting lentoid bodies (g,h) were present still containing γ-crystallin. Often the core of iridal bodies did not contain γ-crystallin any more at this time, but some peripheral regions, apparently newly added to the body were positive for this protein. (a,b) X 110, (c,d) X 97, (e,f) X 108, (g,h) X 104.


a
b
d
f
h

in aggregates. Gamma-crystallin-positive cell clusters have disappeared altogether after 110 days of culture in the high density mixed iris cell cultures (Figure 34), although some single γ-crystallin positive cells have remained. This may be a consequence of lentoid cell death and early suppression of mitotic activity in these cultures.

7.3 CELL CULTURE: CONTINUOUS STIMULATION

In this series of experiments bovine thyrotropin preparation TSH-B8 was constantly present throughout the culture period.

7.3.1 Mitotic Activity. In none of these experiments (Figures 27, 28, 29, 36, 39, 42) did TSH-B8 influence the timing of appearance of the first mitotic figures. This result suggests that TSH-B8 does not control the first event necessary for the stimulation of mitotic activity in dorsal iris epithelial cells. It appears that other cellular events not under the control of TSH-B8 must occur before TSH-B8 can act. This preparation however, greatly elevated the frequency of mitotic figures present during the peak periods. In the high density mixed iris cell culture (Figure 27) the first peak of mitotic activity was almost twice as high in TSH-B8-treated cultures than in controls, but the small third peak of mitosis was comparable in treated and control cultures. In cultures of pure iris epithelial cells the peaks of mitotic activity in the TSH-B8-supplemented cultures were approximately twice as high as those in controls, with the exception of the fourth peak, during which the magnitude of mitotic activity was comparable in experimental and control cultures (Figure 28). In low density mixed cell cultures (Figure 29), the difference between TSH-B8-treated and control cultures was smaller, but still noticeable.

In cultures at high to moderately low cell density maintained in winter to spring time TSH-B8 did not accelerate the appearance of the peaks of mitotic activity (Figures 27, 28, 29). In cultures of moderately to very low cell density (Figures 36, 39, 42) this hormone preparation accelerated the first appearance of mitotic peaks. In control cultures the first mitotic activity peak did not emerge until 35 to 50 days of culture (Figures 36, 39, 42), whereas in continuously TSH-B8-treated cultures the mitotic activity had already reached a maximum at 25 to 30 days (Figures 36, 39 top, 42 bottom). In TSH-B8-treated cultures the first peaks of mitotic activity were twice to several times higher than in controls at the same time of culture (Figures 36, 39 top, 42 bottom). When in the control cultures the peak of mitotic activity was reached after around 40 days of culture, the differences in mitotic activity between treated and control cultures became small, undiscernible (Figures 36, 39 top) or elevated slightly (Figure 42 bottom). Enhanced mitotic activity could be maintained in continuously TSH-B8-supplemented cultures for considerable time periods, for example from days 20 to 40 (Figures 39 top, 42 bottom) or 20 to 60 (Figure 36 top). It

Figure 36. Mitotic activity in very low density mixed iris cell cultures. Stimulation with 3 to 12 µg/ml TSH-B8 (closed symbols) was started on days 0, 4, and 15 after explantation (solid lines), while no TSH-B8 was added to the controls (dashed lines). The time of the addition of TSH-B8 is indicated to the right of the graphs. Iris cell suspensions consisting of epithelial cells, stromal fibroblasts, and iridophores from 5 irises per dish were cultured for 60 days. After preparation of the suspension 6.2 x 10^3 cells were seeded in each dish, and about 3.4 x 10^3 of them attached and spread (55%). Mitotic figures were counted at intervals of 5 days. Each line and symbol represents one culture dish.

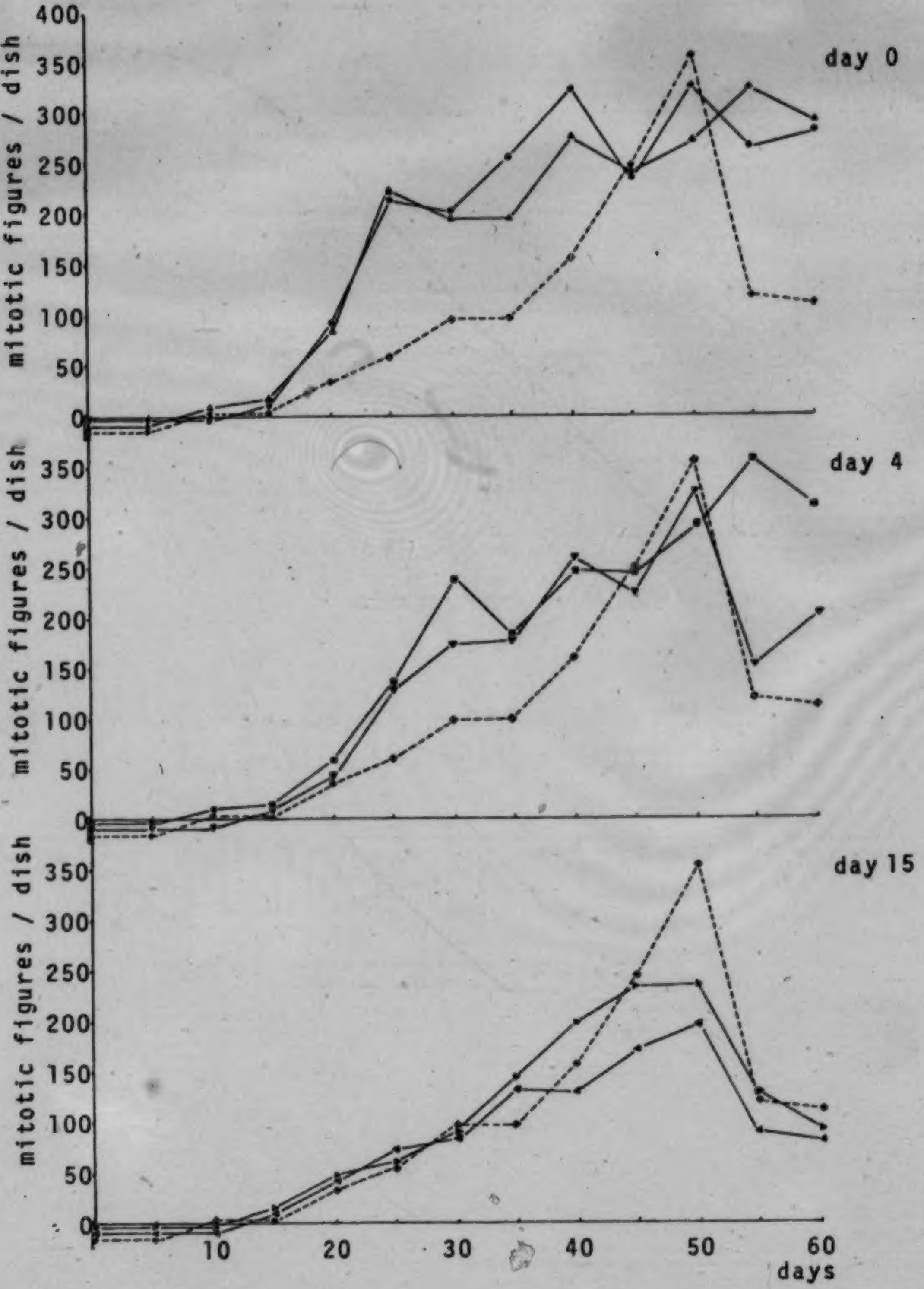

400
350
300
250
200
150
100
50
0
mitotic figures / dish
day 0
350
300
250
200
150
100
50
0
mitotic figures / dish
day 4
350
300
250
200
150
100
50
0
mitotic figures / dish
day 15
10
20
30
40
50
60
days

Figure 37. Iridal bodies in very low density mixed iris cell culture (same culture as in Figure 36). See Figure 36 for further explanations regarding culture conditions. Iridal bodies were counted at 5 day intervals. The time of addition of TSH-B8 is shown to the right of the graphs.

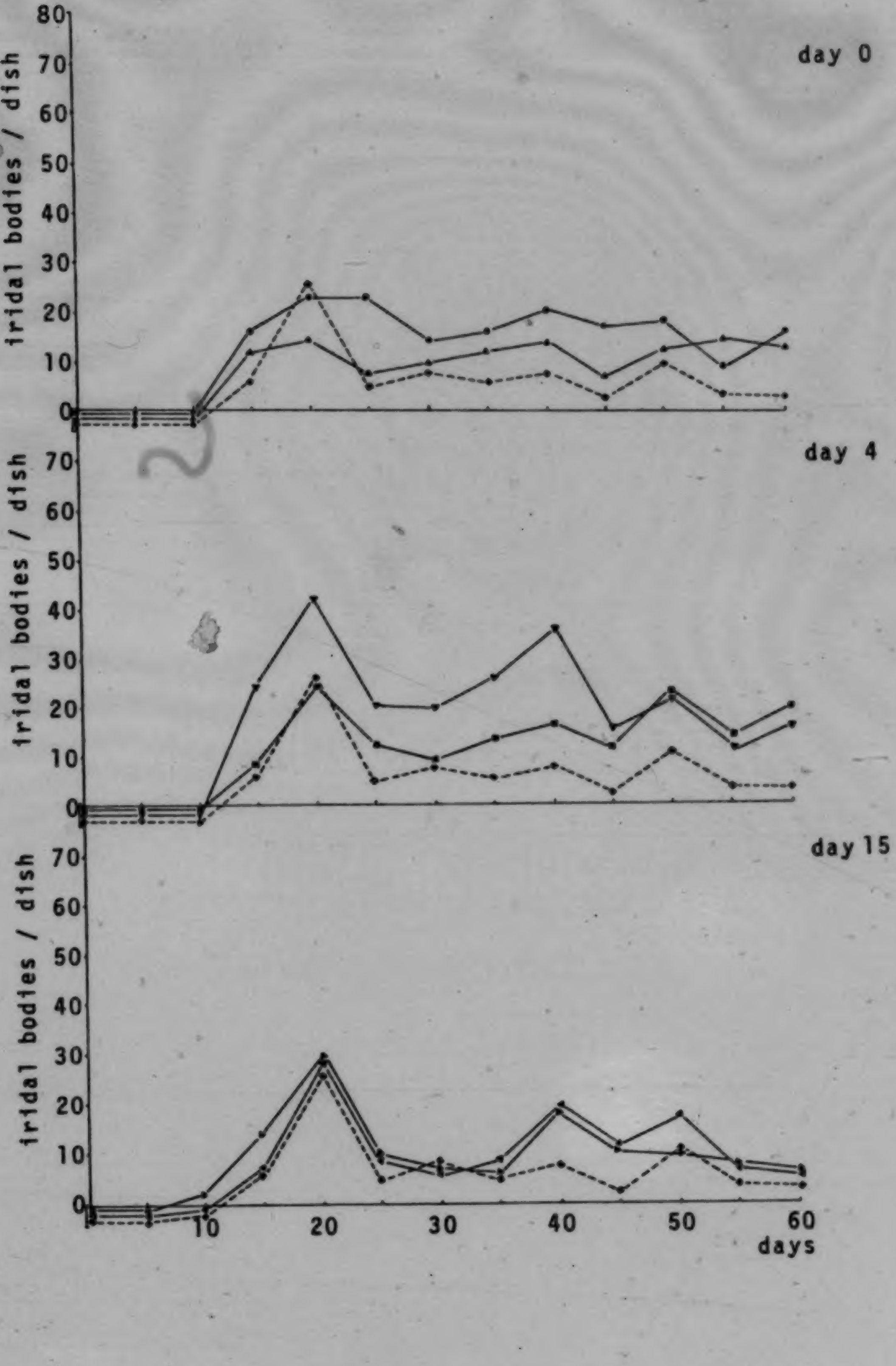
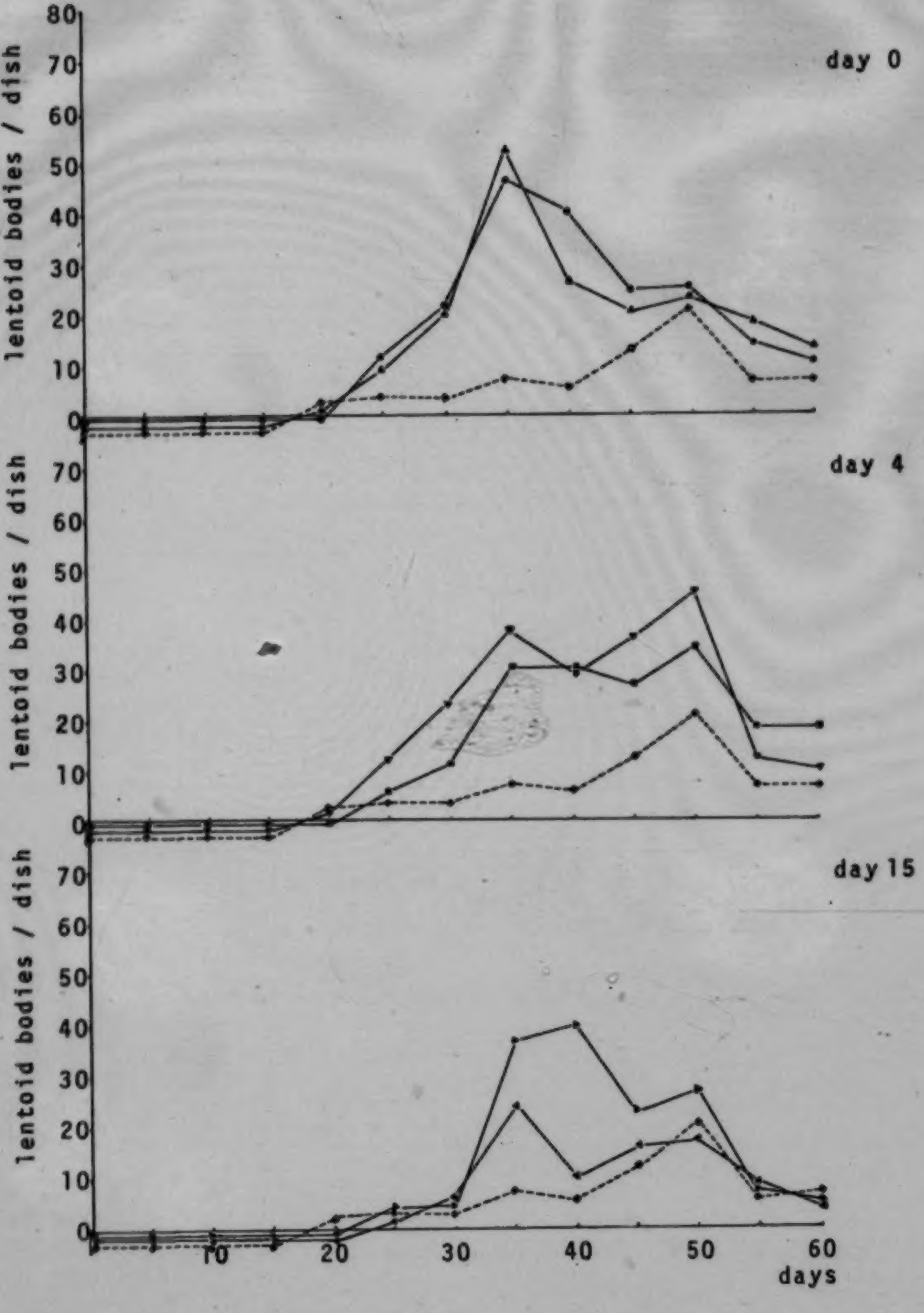

day 0
day 4
day 15
iridal bodies / dish
iridal bodies / dish
iridal bodies / dish
80
70
60
50
40
30
20
10
0
70
60
50
40
30
20
10
0
70
60
50
40
30
20
10
0
10
20
30
40
50
60
days

Figure 38. Lentoid bodies in very low density mixed iris cell culture (same culture as in Figure 36). See Figure 36 for further explanations regarding culture conditions. Lentoid bodies were counted at 5 day intervals. The time of addition of TSH-B8 is indicated to the right of the graphs.


day 0
day 4
day 15
lentoid bodies / dish
lentoid bodies / dish
lentoid bodies / dish
0
10
20
30
40
50
60
70
80
10
20
30
40
50
60
days

is not clear why TSH-B8 accelerates the rise in frequency of mitotic cells in very low cell density cultures in summer time, but not in high to moderately low cell density cultures in spring. This discrepancy may reflect seasonal differences in the physiological state of these cells.

7.3.2 Iridal Bodies. TSH-B8 stimulates cluster formation in iris epithelial cells, but not in stromal fibroblasts (Figures 27, 28, 29, 37, 40, 43). TSH-B8 had no influence on depigmentation of iris epithelial cells (Figure 30) and did not accelerate the onset of clustering (Figures 27, 28, 29, 37, 40, 43). At low cell density the effect of TSH-B8 was hardly noticeable in the first 20 to 40 days (Figures 28, 29, 37, 40, 43), but in the high density cultures (Figure 27) the difference between control and TSH-B8-treated cultures was quite striking right from the beginning of clustering. In these cultures the frequency of iridal bodies was drastically elevated above that of controls. In low density cultures the frequency of iridal bodies in TSH-B8-treated cultures was in general also increased when compared to the controls (Figures 37 top, 40 top, 43 bottom).

7.3.3 Lentoid Bodies. Although TSH-B8 does not accelerate lentoid body formation at the concentrations tested, it augments the frequency of these cell clusters (Figures 27, 28, 29, 38 top, 41 top, 44 bottom). This effect occurs under high and low cell density conditions, and regardless of the presence

Figure 39. Mitotic activity in low density mixed iris cell culture. Stimulation with 3 to 12 μg/ml TSH-B8 (closed symbols) was started on day 0, 4, and 15 after explantation (solid lines). No TSH-B8 was added to the controls (dashed lines). Cell suspensions consisting of iris epithelial cells, stromal fibroblasts, and iridophores from 8.75 irises per dish were cultured for 60 days. In each dish 25 x 10^3 cells were innoculated and about 8.3 x 10^3 of them attached and spread (33%). Mitotic figures were counted at intervals of 5 days. Each line represents one culture dish. The time of addition of TSH-B8 is indicated to the right of the graphs.

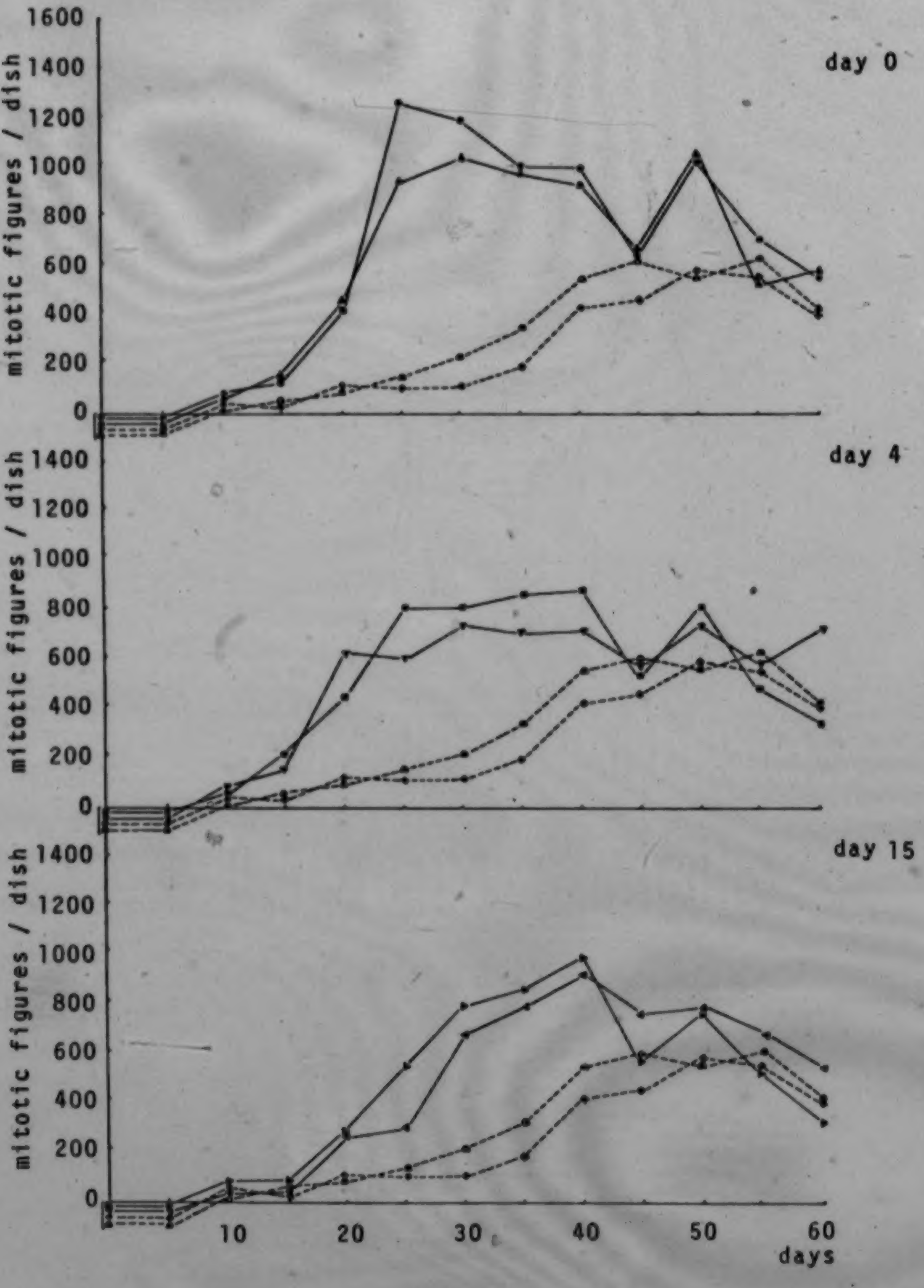

day 0
day 4
day 15
mitotic figures / dish
1600
1400
1200
1000
800
600
400
200
0
10
20
30
40
50
60
days

Figure 40. Iridal bodies in low density mixed iris cell culture (same culture as in Figure 39). See Figure 39 for further explanation regarding culture conditions. Iridal bodies were counted at 5 day intervals. The time of addition of TSH-B8 is indicated to the right of the graphs.

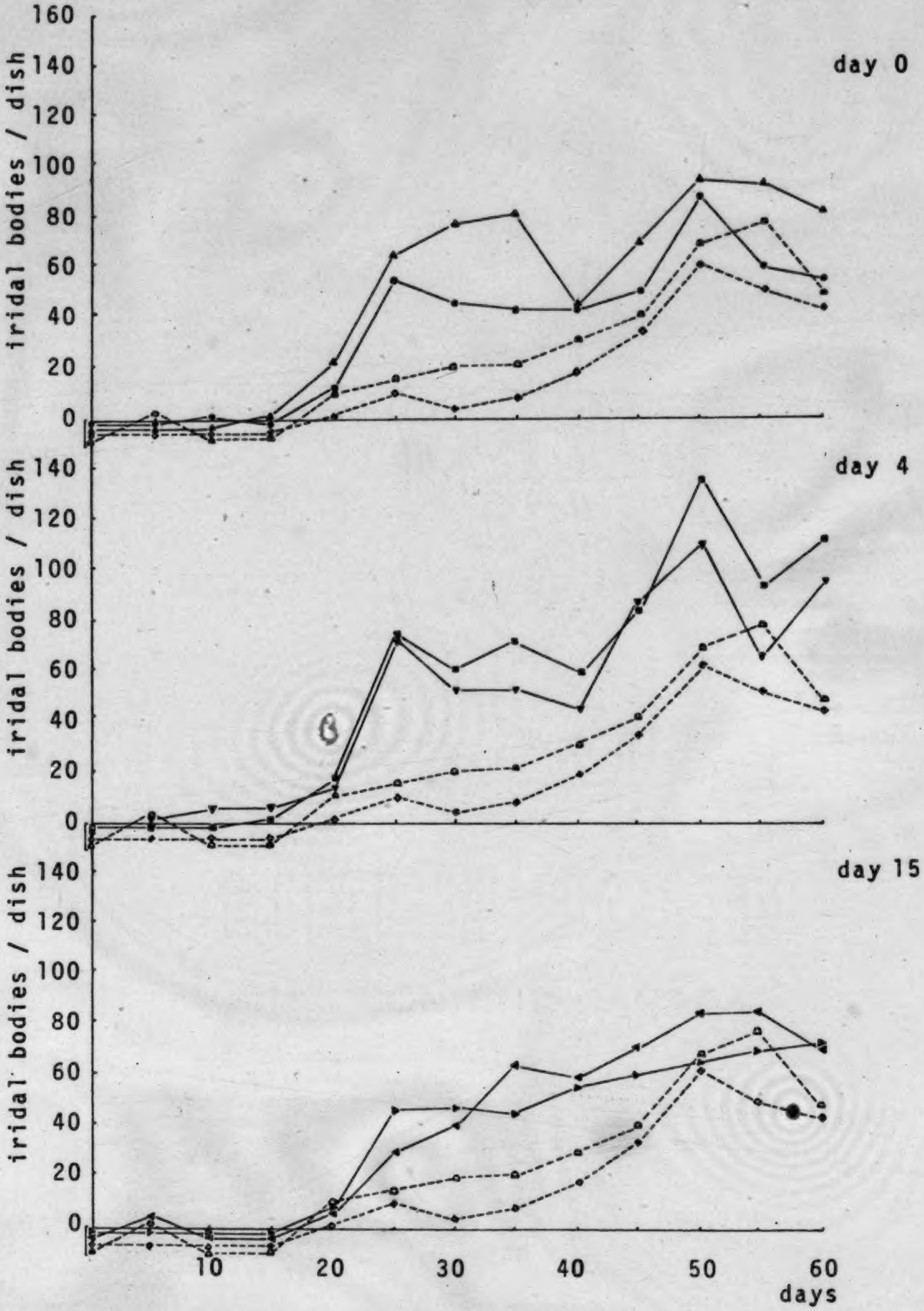
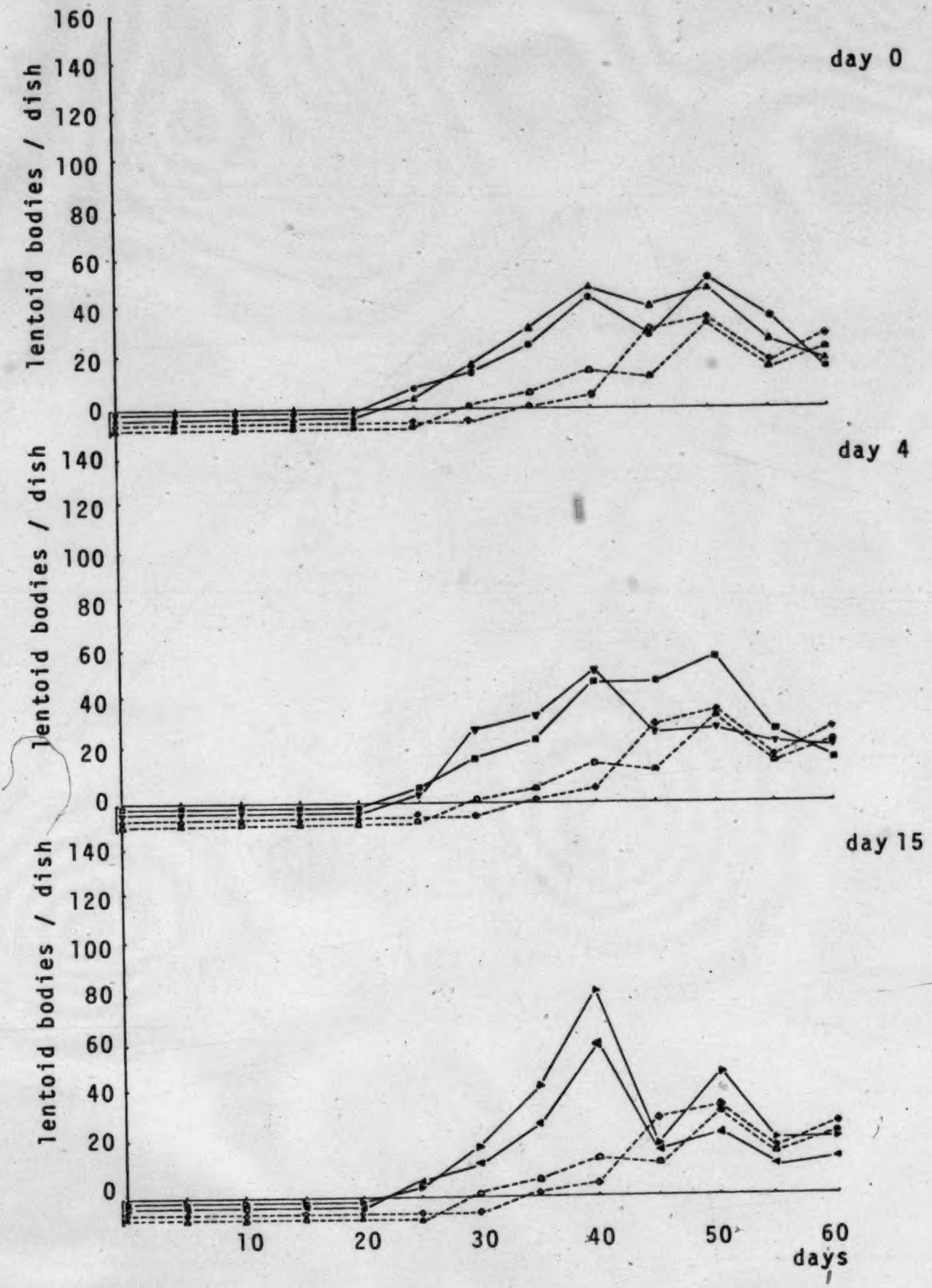

day 0
day 4
day 15
iridal bodies / dish
iridal bodies / dish
iridal bodies / dish
160
140
120
100
80
60
40
20
0
140
120
100
80
60
40
20
0
140
120
100
80
60
40
20
0
10
20
30
40
50
60
days

Figure 41. Lentoid bodies in low density mixed iris cell culture (same culture as in Figure 39). See Figure 39 for further explanations regarding culture conditions. Lentoid bodies were counted at 5 day intervals. The time of addition of TSH-B8 is indicated to the right of the graphs.


day 0
day 4
day 15
lentoid bodies / dish
lentoid bodies / dish
lentoid bodies / dish
160
140
120
100
80
60
40
20
0
10
20
30
40
50
60
days

or absence of stromal fibroblasts. The frequency of lentoid bodies increased approximately 2 to 10 times above the frequency of controls when TSH-B8 was continuously present in high to moderately low cell density cultures (Figures 27, 28, 29).

In continuously stimulated cultures at moderately low to high cell density (Figures 27, 28, 29) a relatively strong first peak of lentoid body formation was already found after 25 to 40 days of culture, and the second maximum followed between days 40 to 60. At a very low cell density, however, this first peak of lentoid bodies was diminished or had disappeared and only the second peak was apparent (Figures 38, 41 top, 44 bottom). This second peak of lentoid body frequency was several times higher than values in controls at the same time of culture (Figures 38 top, 44 bottom).

7.3.4 Presence of Crystallins. In TSH-B8-supplemented cultures lens specific proteins emerged at the same time as in control cultures; after 25 days a crystallin different from γ-crystallin was present and after 50 days γ-crystallin was also present (Figures 34, 35). During embryonic development of the lens in the newt β-crystallins emerge first (McDevitt and Brahma, 1981), and it could be that after 25 days of cell culture β-crystallins also appear before the other crystallins. Since more lentoid bodies and lentoid cells were present in TSH-B8-treated cultures it can be assumed that the total γ-crystallin content of a TSH-B8-stimulated iris cell population was also enhanced. When cultures were examined at a time of low mitotic and clustering activity, for example after 60 days (Figure 45), γ-crystallin-positive clusters were less to slightly more frequent in TSH-B8-treated than in control cultures. Single cells positive for γ-crystallin increase in number during the culture period up to 100 days (Figure 34) in TSH-B8-supplemented cultures.

7.4 CELL CULTURE: DELAYED STIMULATION

In this series of experiments thyrotropin preparation TSH-B8 was added 4 to 15 days after explantation.

7.4.1 Mitotic Activity. In very low density mixed iris cell cultures where TSH-B8 treatment had been started at day 4 after explantation, mitotic activity still rose above control levels in a similar fashion as in continuously stimulated cultures (Figures 36 middle, 39 middle). In the experiments where treatment was started at 15 days this rise was not observed (Figure 36 bottom). In cultures where treatment with TSH-B8 was delayed until day 4, the stimulating effect of this preparation appeared at 15 to 25 days of culture (Figures 36 middle, 39 middle). In cultures where treatment had been started 15 days after explantation, the stimulatory effect on mitosis was first observed on day 20 to 35 of culture (Figures 36, 39 bottom).

In cultures in which treatment started on day 4, the mitotic activity in the first peak was markedly higher than in controls (Figures 36, 39 middle). In cultures treated from day 15 onwards this hormone preparation had a variable effect ranging from several times the activity of controls to no effect (Figures 36, 39 bottom). The presence of TSH-B8 seems to be unimportant in the first four days of culture, since the later rise in mitotic activity observed in continuously stimulated cultures also occurred if stimulation was delayed by four days.

7.4.2 Iridal Bodies. Where stimulation had been delayed to the fourth day, the frequency of iridal bodies still rose on days 15 to 20 of culture (Figures 37, 40 middle), and the clustering pattern, in general, resembled the one in continuously stimulated cultures (Figures 37, 40 top). The frequency of iridal bodies was clearly higher than in the controls (Figures 37, 40 middle). When stimulation was delayed up to the day 15 of cultivation, the clustering frequency did not rise above control levels until days 25 to 40 (Figures 37, 40 bottom); maximally, it was 3.5 to 5 times higher in TSH-B8-treated cultures (Figures 37, 40 bottom).

7.4.3 Lentoid Bodies. In cultures which had been treated with TSH-B8 with a delay of 4 or 15 days, lentoid body formation reached its peak around the same time, 40 days, as in continuously stimulated cultures. When compared to the controls at the same time, clustering peaks attained four times to five times higher heights in the case of 4 days (Figures 38, 41 middle), and eight times (Figures 38, 41 bottom) higher heights in the case of 15 days delayed stimulation. In one of the two experiments (Figure 38 bottom) lentoid body formation was repressed up to day 30 after 15 day-delayed stimulation. Similarly, in cultures in which stimulation was delayed for 4 days the first peak of lentoid frequency appeared on about day 35 (Figure 38 middle). Lentoid body formation was not delayed by the same length of time as the delay of addition of TSH-B8. When treatment was deferred until day 15 a higher inconsistency in the responses seemed to exist, but absence of TSH-B8 in the first 4 days of culture seemed to have no unfavorable effects on the lentoid body formation.

7.4.4 Presence of γ-Crystallin. In regards to the presence of γ-crystallin after 60 days of culture, cultures where treatment was delayed for 4 days were similar to continuously treated cultures, whereas cultures treated from day 15 onwards appeared to be more similar to controls at fixing time (Figure 45 left and center). Hence, omitting TSH-B8 treatment in the first 4 days of cultivation had little influence on the γ-crystallin pattern at 60 days, but deferring of the treatment until day 15 decreased the occurrence of γ-crystallin in these cultures.

7.5 CELL CULTURE: WITHDRAWAL OF STIMULATION

In this series of experiments thyrotropin preparation TSH-B8 was withdrawn 4 or 15 days after explantation.

7.5.1 Mitotic Activity. In very low density mixed iris cell cultures which had been supplied with TSH-B8 only for the first 4 days of culture the mitotic activity matched closely the activity in controls (Figure 42 top). In cultures that had been supplemented with the TSH-B8 preparation for the first 15 days of cultivation, the mitotic activity of the TSH-B8-treated cultures reached twice the value of controls at 20 days of culture. After this period aberrations were obtained; the first peak of mitotic activity occurring between day 30 and 40 was actually decreased to half when compared to the controls, and by 45 days nonconsistent results were obtained in the experimental treatments. The results obtained using interrupted stimulation with TSH-B8 show that TSH-B8 does not act as a trigger, but rather as a trophic stimulator, which must be continuously present after day 4 of culture to support elevated mitotic activity.

7.5.2 Iridal Bodies. In cultures which were stimulated with TSH-B8 for the first 4 to 15 days only, the frequency of iridal bodies was very similar to the frequency in control cultures (Figure 43), although in the cultures stimulated for the first 4 days there were slightly more iridal bodies. In cultures that had been supplemented with this preparation for the first 15 days, the frequency of iridal bodies increased above control levels around the 25th day of culture. It appears from these data that TSH-B8 stimulates trophically the iris epithelial cells, at least from day 4 onward, so that they

Figure 42. Mitotic activity in very low density mixed iris cell culture. The hormone preparation TSH-B8 at a concentration ranging from 3 to 12 μg/ml, (closed symbols) was withdrawn (open symbols) on days 4, 15 and 60 after explantation (solid lines), and no TSH-B8 was added to the controls (dashed lines). Cell suspensions consisting of iris epithelial cells, stromal fibroblasts, and iridophores from 5 irises per dish were cultured for 60 days. In each dish 12 x 10^3 cells were innoculated, and about 2.3 x 10^3 of them attached and spread (19%). Mitotic figures were counted at intervals of 5 days. Each line and symbol represents one culture dish. The time of withdrawal of the hormone preparation is indicated to the right of the graphs.

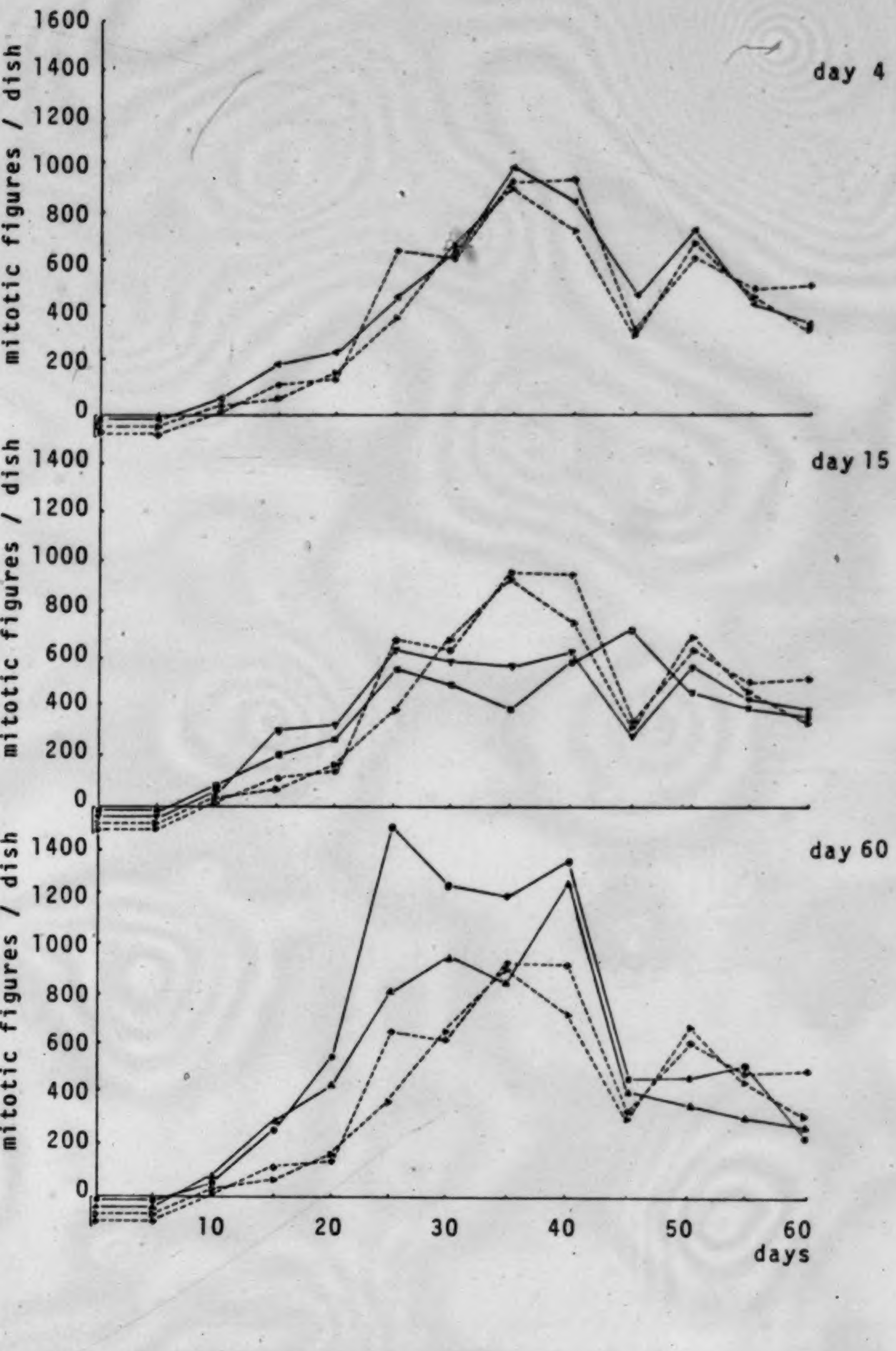

day 4
day 15
day 60
mitotic figures / dish
1600
1400
1200
1000
800
600
400
200
0
10
20
30
40
50
60
days

Figure 43. Iridal bodies in very low density mixed iris cell culture (same culture as in Figure 42). See Figure 42 for further explanation regarding culture conditions. Iridal bodies were counted at 5 day intervals. The time of withdrawal of the hormone preparation is indicated to the right of the graphs.

7

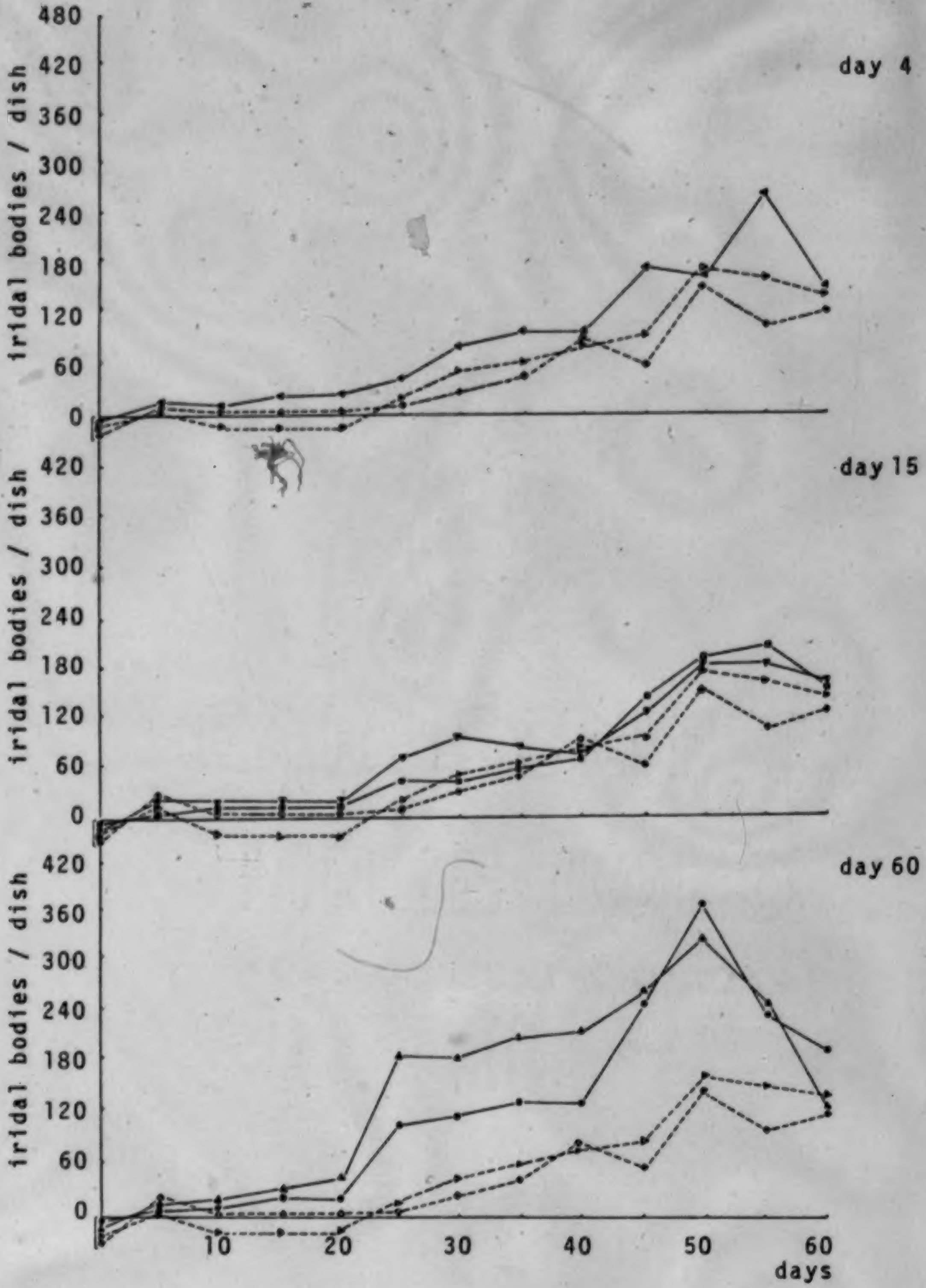
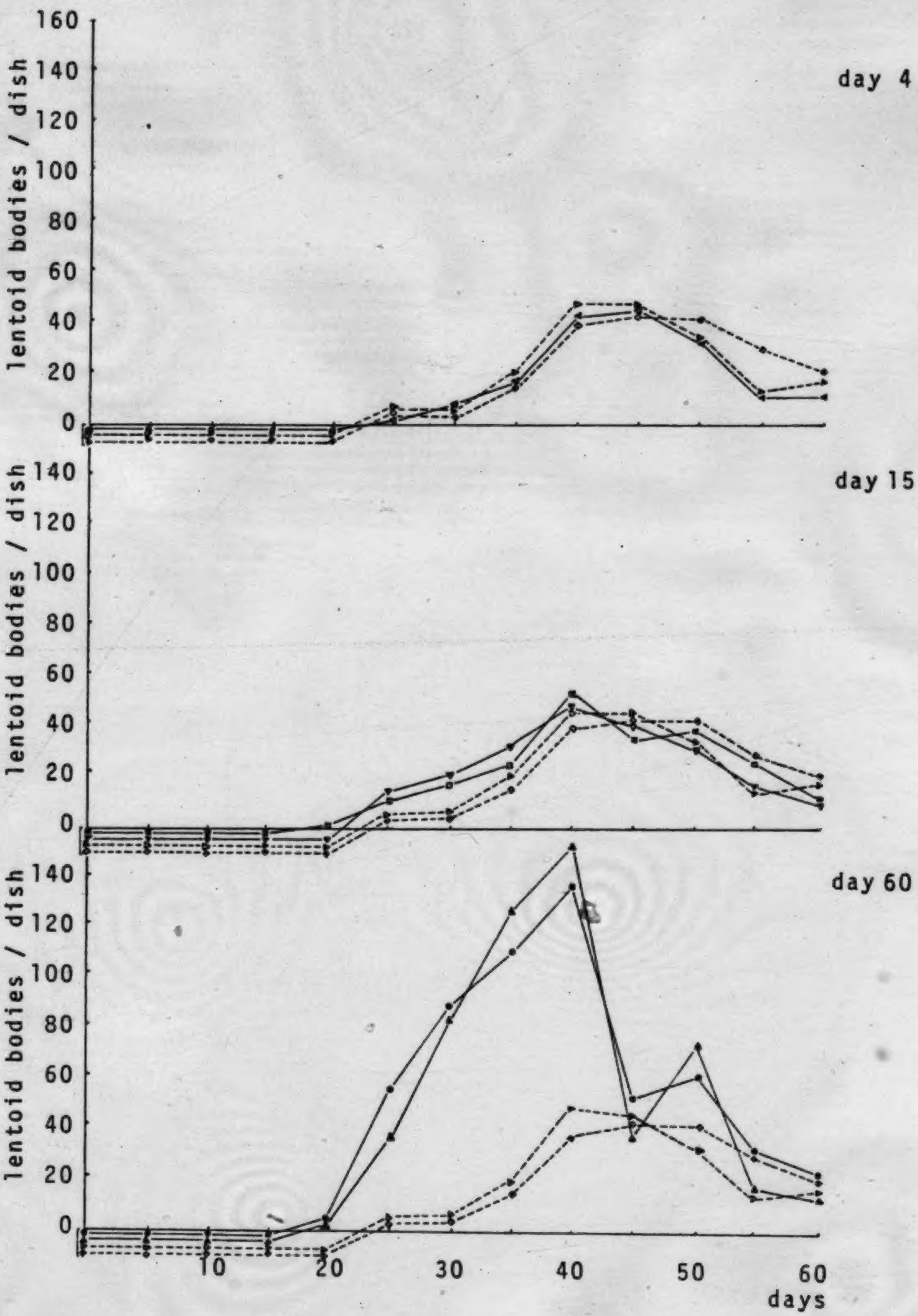

480
420
360
300
240
180
120
60
0
iridal bodies / dish
day 4
420
360
300
240
180
120
60
0
iridal bodies / dish
day 15
420
360
300
240
180
120
60
0
iridal bodies / dish
day 60
10
20
30
40
50
60
days

Figure 44. Lentoid bodies in very low density mixed iris cell culture (same culture as in Figure 42). See Figure 42 for further explanation regarding culture conditions. Lentoid bodies were counted at 5 day intervals. The time of the withdrawal of the hormone preparation is indicated to the right of the graphs.

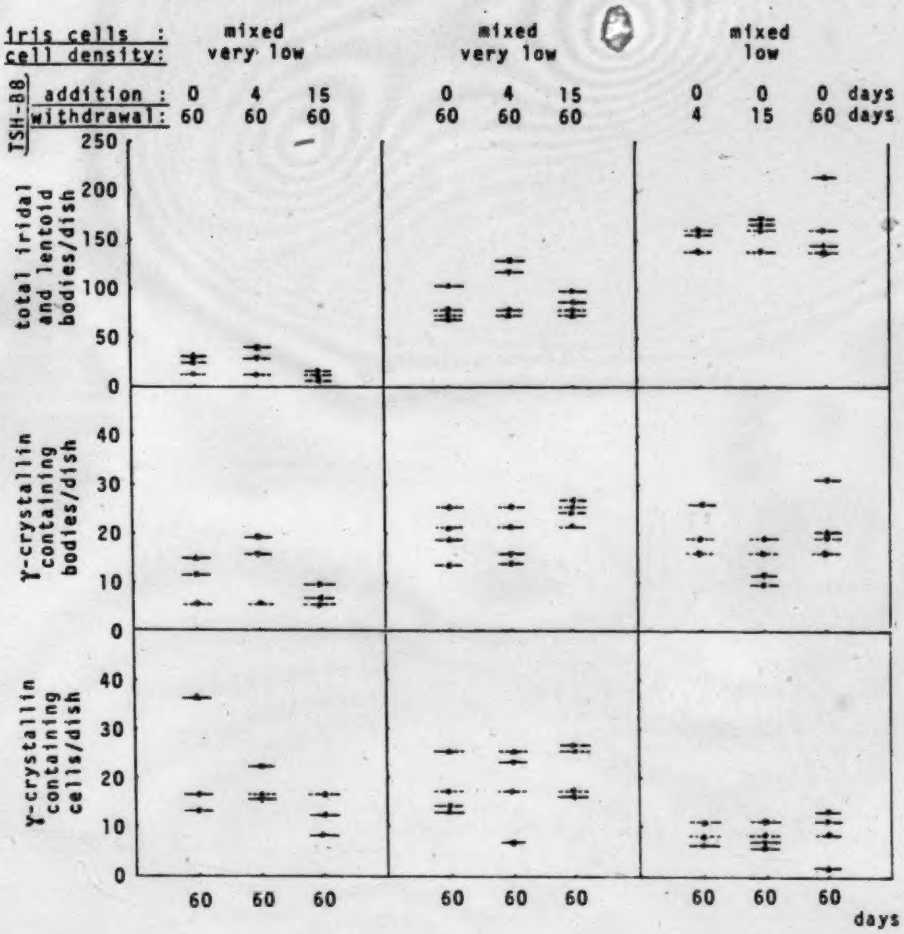

lentoid bodies / dish
160
140
120
100
80
60
40
20
0
day 4
lentoid bodies / dish
140
120
100
80
60
40
20
0
day 15
lentoid bodies / dish
140
120
100
80
60
40
20
0
day 60
10
20
30
40
50
60
days

organize into a larger number of iridal bodies.

7.5.3 Lentoid Bodies. Cell cultures in which TSH-B8-treatment was stopped at day 4 had the same number of lentoid bodies as control cultures, and the frequency curves of lentoid bodies of the two treatment groups followed each other closely (Figure 44 top). When the treatment was stopped at day 15, the frequency of lentoid bodies at day 30 was higher in TSH-B8-treated cultures than in controls (Figure 44 middle). At this time, in continuously stimulated cultures, the frequency of lentoid bodies in TSH-B8-treated cultures was many times higher than in the controls (Figure 44 bottom). In cultures treated for the first 15 days the small rise of lentoid bodies above control levels lasted from day 25 to 35, and later no differences were found between control and treated groups. It is possible that the increase in lentoid bodies observed in 15 day stimulated cultures, 10 to 15 days after withdrawal of TSH-B8, represents a delayed response to the treatment. It appears from these results that the presence of TSH-B8 is most important at the time when the iris epithelial cells exhibit a trend to form lentoid bodies.

7.5.4 Presence of γ-Crystallin. After 60 days of cultivation, cultures treated for the first 4 or 15 days only (Figure 45 right) had either lower or similar frequencies of γ-crystallin containing clusters or single cells when compared to controls. This was expected from the data on Figure 44.

Figure 45. Presence of γ-crystallin after 60 days in cell cultures with or without TSH-B8 at a concentration varying in each dish from 3 to 12 μg/ml. Left and center, two replicate very low density mixed iris cell cultures (left, same as Figures 36 to 38; center, same as Figures 39 to 41). In these cultures TSH-B8 treatment has been delayed for 0, 4, or 15 days after explantation. When these cultures were examined after 60 days they were in a phase of low mitotic and clustering activity. Right, low density mixed iris cell cultures where TSH-B8 was withdrawn after 4, 15, or 60 days of culture (right, same as Figures 42 to 44). All culture dishes were fixed and stained with fluorescein labelled antibody for the detection of γ-crystallin. Treated dishes - solid lines, control dishes - dashed lines; dishes still treated at time of fixation - solid symbols, dishes not treated at time of fixation - open symbols. The different symbols identify individual dishes in experiments (Figures 36 to 44). The total amount of clusters (top of graph) is the sum of iridal and lentoid bodies (Figures 37, 38, 40, 41, 43, 44). Gamma-crystallin was present in lentoid and some iridal bodies, and also some single cells (center and bottom).


iris cells :
cell density:
mixed
very low
mixed
very low
mixed
low
TSH-B8
addition :
withdrawal:
0 4 15
60 60 60
0 4 15
60 60 60
0 0 0 days
4 15 60 days
total iridal and lentoid bodies/dish
0
50
100
150
200
250
γ-crystallin containing bodies/dish
0
10
20
30
40
γ-crystallin containing cells/dish
0
10
20
30
40
60 60 60
60 60 60
60 60 60
days

8. EFFECT OF BOVINE THYROTROPIN PREPARATION TS-10

8.1 ORGAN CULTURE

8.1.1 Regeneration Stages. Dorsal irises were cultured in the presence of the bovine thyrotropin preparation TS-10 at concentrations ranging from 12 to 1400 μg/ml (Figure 46). Only the highest concentration of this preparation elicited a significant percentage of lens regeneration, with irises developing beyond the depigmentation stage 2, in 43% (6 of 14) of the cases compared to 11% (2 of 19) in the controls. At concentrations of 14 and 12 μg/ml no significant stimulation of lens regeneration occurred (Figure 46). The frequencies of lens specific regeneration stages were 8% (1 of 12) and 6% (1 of 17) respectively in the 14 and 12 μg/ml TS-10-supplemented cultures, compared to 11% (2 of 19) and 6% (1 of 17) in their respective controls.

After culture of dorsal irises in the presence of 1400 μg/ml TS-10, the most advanced lens regenerate obtained was a large lens vesicle judged to be at stage 5. In this case, however, the majority of its epithelial cells had an abnormally high degree of pigmentation and only a cluster of depigmented cells resembling stage 3 was apparent at the original pupillary margin of the iris (Figure 47). The other lens vesicle-like structures in this group which were classified as belonging to stage 4, were also pigmented, one of them rather heavily. However, they were of relatively normal size and shape. Depigmentation stage 2, and depigmented pupillary margin stage 3

Figure 46. Lens regeneration stages of dorsal irises after 20 days of culture maintained with or without the bovine thyrotropin preparation TS-10; 14 μg/ml TS-10 = 0.01 I.U./ml. The TS-10 used in these experiments had been stored dry at -20°C for about eighteen months prior to use. No significant frequency differences were found (Chi square) between controls and TS-10-treated cultures, except at the highest concentration of this preparation (1400 μg/ml): $^*P < 0.05$. No γ-crystallin could be visualized with the immunofluorescence method. See Figure 6 for further explanation of the histograms and Figure 7 for explanation of the + and - symbols.

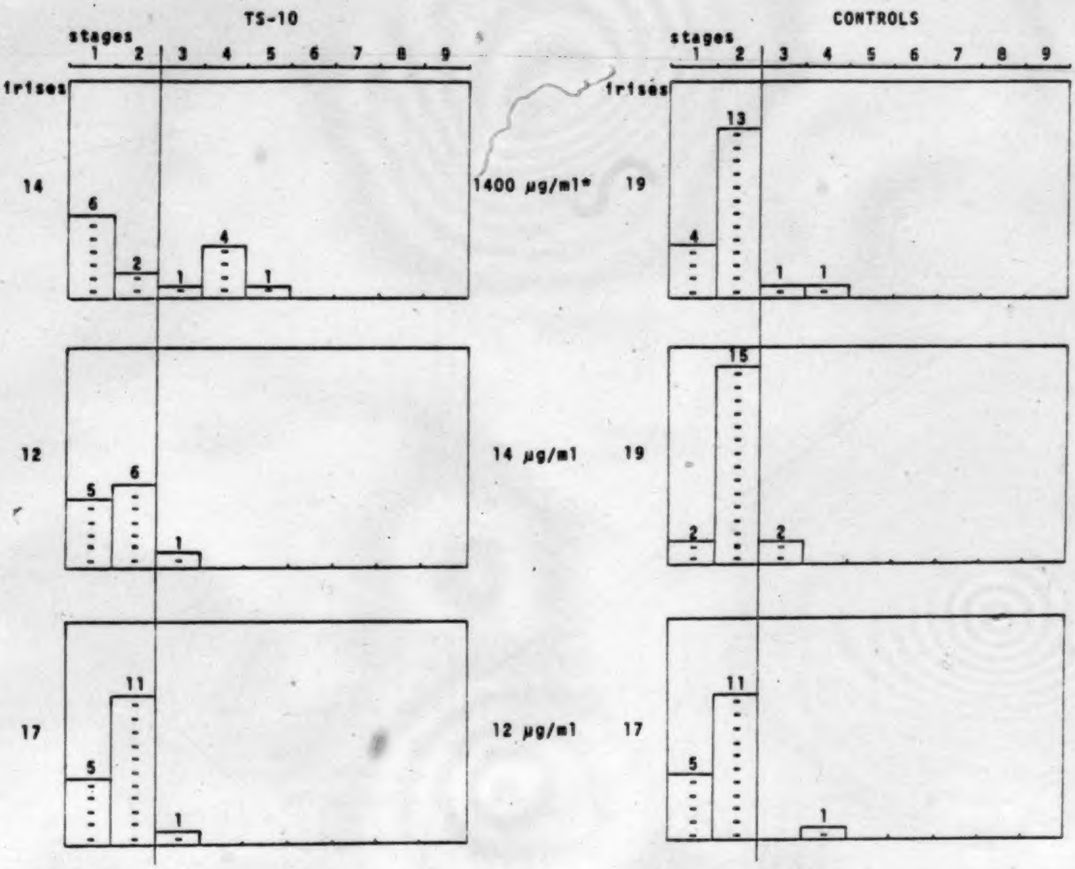

TS-10
CONTROLS
stages
1 2 3 4 5 6 7 8 9
irises
14
6
2
1
4
1
1400 µg/ml*
19
4
13
1
1
12
5
6
1
14 µg/ml
19
2
15
2
17
5
11
1
12 µg/ml
17
5
11
1

Figure 47. An abnormally heavily pigmented lens vesicle with a shape similar to that of a stage 5 regenerate, which developed on a dorsal iris after 20 days of culture in the presence of 1400 μg/ml of TS-10. At the pupillary margin (arrow) a group of thickened depigmented cells, resembling those found at stage 3, is visible. The inner lamina of the iris epithelium has depigmented in some regions, as is normally found in stages 2. The lumen of the lens vesicle is filled with an atypically large pigmented mass of condensed cell debris. X 326. Stained with hematoxylin-eosin. No γ-crystallin could be detected in this lens regenerate.

were less or equally frequent as in controls, but the totally pigmented iris swelling stage 1 was very abundant. Although the formation of a lens-like pattern was stimulated by TS-10, the conversion of cell type from pigmented iris epithelial cells to lens cells was delayed or suppressed. That an inhibition of depigmentation occurred in TS-10-treated cells is also supported by the relatively high frequency of early regenerates; in the group treated with a high concentration of TS-10 irises belonging to stage 1 were more frequent than irises at stage 2 and also when compared to the controls.

8.1.2 Presence of γ-Crystallin. γ-Crystallin, a marker for lens fiber cell differentiation, was not detected in lens regenerates in TS-10-supplemented cultures (Figures 46, 47), suggesting that cell type conversion had not progressed very far.

9. EFFECT OF OVINE THYROTROPIN PREPARATION oTSH-9

9.1 ORGAN CULTURE

9.1.1 Regeneration Stages. Dorsal irises were cultured in the presence of ovine thyrotropin preparation oTSH-9 at two concentrations, 1.4 and 0.5 μg/ml (Figure 48). A highly significant number of lens regenerates formed in cultures supplemented with 1.4 μg/ml of this preparation. In these cultures, 77% (10 of 13) of the irises had developed lenses at regeneration stages 3 or higher; this contrasts with a frequency of only 11% (2 of 19) for the controls. The lower

Figure 48. Lens regeneration stages of dorsal irises after 20 days of culture with or without the ovine thyrotropin preparation oTSH-9; 1.4 μg/ml oTSH-9 = 0.01 I.U./ml. This hormone preparation had been stored dry at -20°C for about six months prior to use. The frequency of specific lens regeneration stages (stages 3 and higher) is significantly higher in the oTSH-9-treated cultures containing 1.4 μg/ml of this hormone preparation. ****$P < 0.0005$. The presence of γ-crystallin was confirmed by immunofluorescence in regenerates at stages higher than 5 and in a single iris at stage 4 in oTSH-9-treated irises. Controls were negative for this protein. See Figure 6 for further explanation of the histograms and Figure 7 for explanation of the + and - symbols.

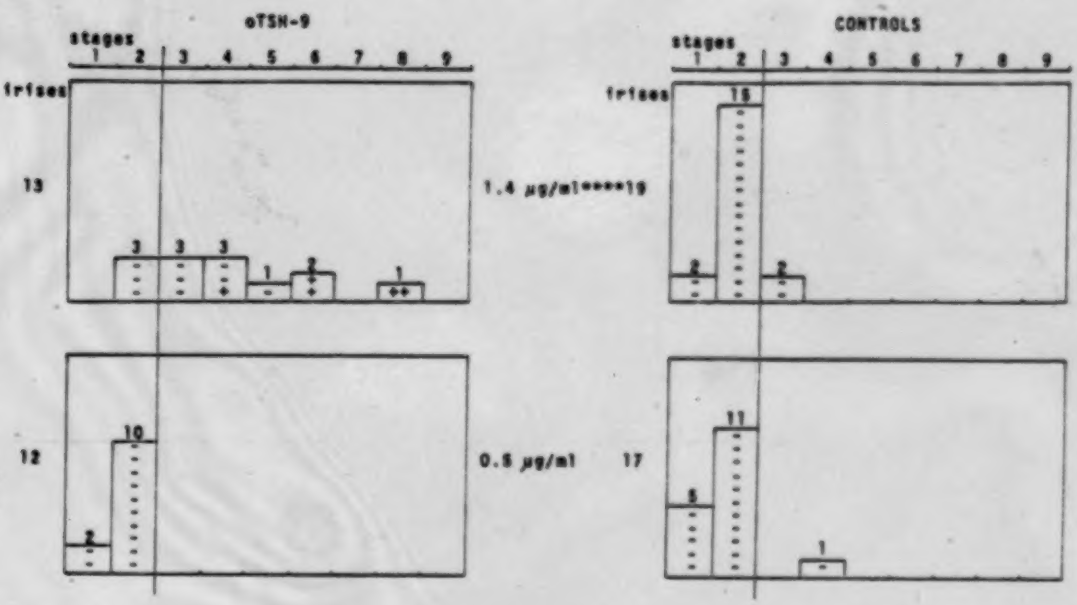

oTSH-9
CONTROLS
stages
1
2
3
4
5
6
7
8
9
irises
13
3
3
3
1
2
1
1.4 µg/ml*****19
2
15
2
12
2
10
0.5 µg/ml
17
5
11
1

Figure 49. Gamma-crystallin accumulation in a lens regenerate of stage 8 which developed on a dorsal iris after 20 days of culture in the presence of 1.4 μg/ml oTSH-9. (a) Stained transmission light micrograph, and (b) corresponding immunofluorescent light micrograph stained with anti-frog γ-crystallin antiserum. (a,b) X 471. Note that the lens epithelium-like cells are not stained (arrow).

a
b

concentration of this hormone preparation (0.5 μg/ml) did not elicit any specific lens regeneration response (Figure 48). This latter result appears extreme. It is possible that if a larger experimental sample could have been prepared some advanced lens regenerates could have been found in this case.

The most advanced lens regenerate found in cultures supplemented with 1.4 μg/ml of oTSH-9 was a small but characteristically shaped lens with a primary fiber core and showing the beginning secondary fiber elongation (Figure 49). In the controls, only two irises exhibited thickened depigmented pupillary margins characteristic of stage 3. Only depigmentation stage 2, compared to an early lens vesicle at stage 4 in controls, was reached by irises cultured in the presence of 0.5 μg/ml oTSH-9.

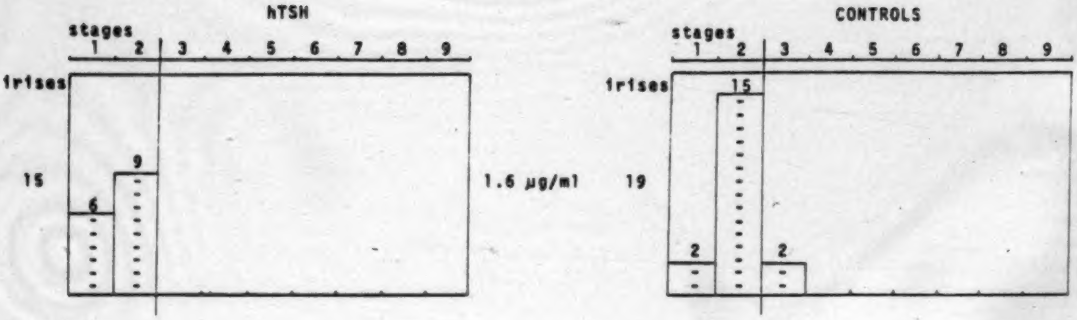
9.1.2 Presence of γ-Crystallin. As would be expected from experience gained *in vivo* (McDevitt and Brahma, 1979), γ-crystallin is present in all lenses at a more advanced regeneration stage than stage 5 (Figures 48, 49). One of the lens vesicles judged to be at stage 4, contained an occasional γ-crystallin-positive cell. In controls, γ-crystallin could not be revealed.

10. EFFECT OF HUMAN THYROTROPIN PREPARATION hTSH

10.1 ORGAN CULTURE

10.1.1 Regeneration Stages. The human thryotropin preparation hTSH did not significantly stimulate lens regeneration at the

Figure 50. Lens regeneration stages of dorsal irises after 20 days of culture with or without the human thyrotropin preparation hTSH; 1.6 μg/ml hTSH = 0.008 I.U./ml, as determined by radioimmunoassay. This preparation was used immediately after its arrival. No significant differences were found (Chi square) between controls and treated cultures at this concentration. No γ-crystallin could be visualized with the immunofluorescence method. See Figure 6 for further explanation of the histograms and Figure 7 for explanation of the + and - symbols.


hTSH
stages
1
2
3
4
5
6
7
8
9
irises
15
6
9
1.6 µg/ml
CONTROLS
stages
1
2
3
4
5
6
7
8
9
irises
19
2
15
2

only concentration tested, 1.6 µg/ml (Figure 50). No specific lens regenerates had formed by the end of the culture period of 20 days, although in the control cultures a few depigmented thickened pupillary margins were noticed.

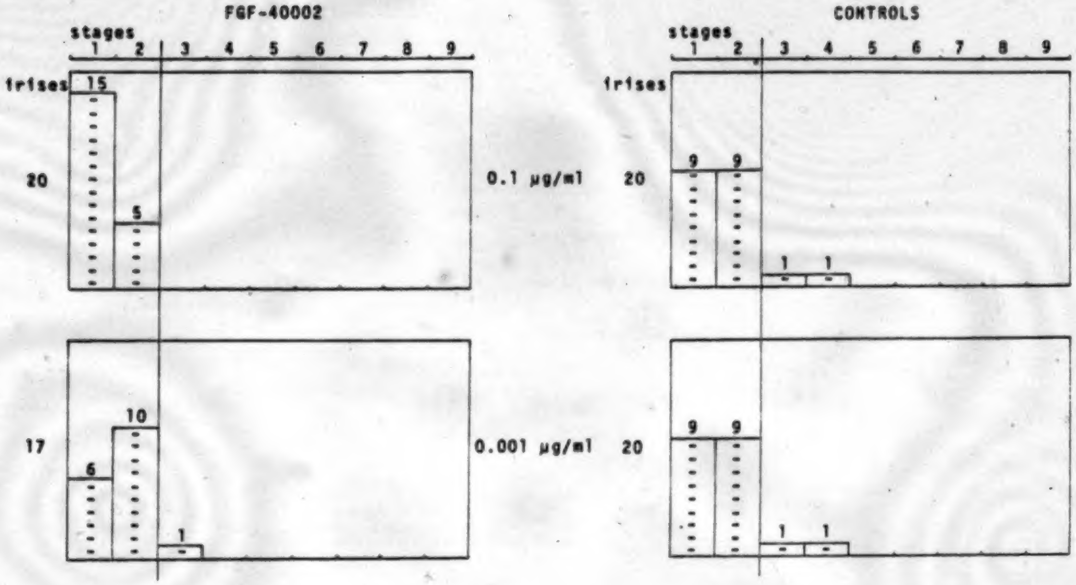
10.1.2 Presence of γ-Crystallin. No lens fiber cells and hence no γ-crystallin-positive cells were present in any of the irises.

11. EFFECT OF BOVINE PITUITARY FIBROBLAST GROWTH FACTOR PREPARATION FGF-40002

11.1 ORGAN CULTURE

11.1.1 Regeneration Stages. Pituitary fibroblast growth factor activity has been reported to be a contaminant in preparations of bovine pituitary hormones, in particular lutropin and thyrotropin (Gospodarowicz et al., 1975), and this factor stimulated mitosis in cultured bovine lens epithelia (Gospodarowicz et al., 1977). The preparation FGF-40002 of bovine pituitary fibroblast growth factor did not stimulate lens regeneration in the newt at concentrations ranging from 0.001 to 0.1 µg/ml (Figure 51). At the highest concentration no specific lens regeneration stages were attained after 20 days of culture, although in a control culture an early lens vesicle of stage 4 was found. At the highest concentration tested in this experiment, 0.1 µg/ml, this preparation appeared to be cytotoxic, since in many irises no nuclear enlargement indicative of cell activation at stage 1, or mitotic activity could be observed. Stromal cells had dis-

Figure 51. Lens regeneration stages of dorsal irises after 20 days of culture with or without bovine pituitary fibroblast growth factor FGF-40002. Purity: 88% by sodium dodecyl sulfate polyacrylamide gel electrophoresis. It was stated by the manufacturer that this preparation could stimulate incorporation of ^{3}H-thymidine in cell cultures of BALB/c 3T3 mouse fibroblasts. Lens regeneration was not significantly different (Chi square), and instead the higher FGF-40002 concentration seemed to be slightly inhibitory. No γ-crystallin was detected with immunofluorescence. See Figure 6 for further explanation of the histograms and Figure 7 for explanation of the + and - symbols.


FGF-40002
CONTROLS
stages
1 2 3 4 5 6 7 8 9
irises
15
5
20
9
9
1
1
0.1 µg/ml
20
10
6
1
17
0.001 µg/ml
20
9
9
1
1

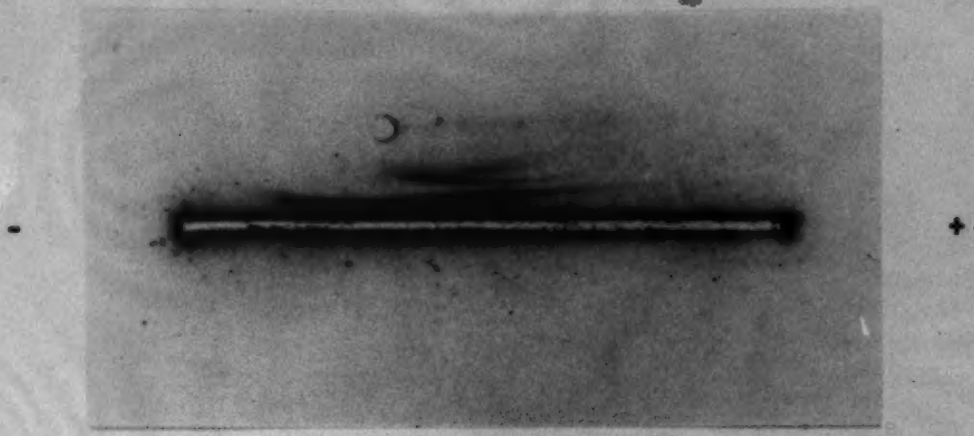
appeared completely in some of the irises, leaving only a mesh of stromal extracellular matrix.

11.1.2 Presence of γ-Crystallin. No γ-crystallin-positive cells were present in any of the irises.

12. AN ATTEMPT TO IDENTIFY PITUITARY GLYCOPROTEIN-LIKE COMPOUNDS IN THE FROG RETINA

Like the pituitary gland, the frog retina has been demonstrated to produce a lens regeneration stimulating factor (Yamada et al., 1973). There are two possibilities arising from the above mentioned findings. The pituitary gland and the retina produce the same stimulating substance, or two different substances are produced in these tissues and both can stimulate lens regeneration from dorsal iris cells. The ovine thyrotropin preparation oTSH-9 was found in this work to stimulate lens regeneration in the newt. This hormone preparation had been initially extracted from pituitary glands using aqueous mixtures of 40% ethanol and 6% ammonium acetate and precipitated with an aqueous mixture of 80% ethanol and 2% ammonium acetate (Parlow, 1979). This same procedure has been used in frog pituitary glands for the isolation of glycoproteins (Licht and Papkoff, 1974) from which a thyrotropin fraction was obtained (McKenzie and Licht, 1978). It seemed worthwhile to prepare an antiserum against this extracted fraction from frog pituitary glands (*Rana pipiens*), and to determine in normal and lentectomized eyes if the retina contained any molecules that could cross-react with this antiserum. In 12% polyacrylamide disc gel electrophoresis the pituitary glycoprotein fraction gave rise to four bands (data not shown). When the antiserum was tested against this fraction by immunoelectrophoresis it gave rise to three major precipitation arches (Figure 52). One of these arches was long and had several lobes possibly due to molecular heterogeneity of the antigen. When the specificity of this antiserum was tested in cross sections of the eye of the frog (*Rana pipiens*) it stained corneal and lens epithelial cells, and a fibrous-looking element in the adult lens fiber cells. It also stained weakly vitreous material, especially in lentectomized eyes. This may be correlated with the general rise in protein content in the vitreous chamber (Gulati and Reyer, 1980) which was observed in the newt after lens removal. No cells were stained in sections of the normal neural retina, but the inner limiting basement membrane was highly fluorescent, suggesting that the antiserum was reacting with some extracellular matrix protein of this tissue. No cross-reaction with human cold-insoluble fibronectin was found when this antiserum was tested against this glycoprotein using a ring test. In one frog eye at five days after lentectomy (Figure 53), the endings of the branched inner processes of the Müller cells contained a material cross-reacting with the antiserum, but the inner limiting basement membrane of the neural retina had been shed. It was present in the vitreous chamber and still stained with the antiserum. As opposed to retinal astrocytes or ganglion cells, which are aligned parallel to the inner limiting basement membrane in the innermost portion of the neural retina (Büssow, 1980), the Müller cells are arranged radially and they send out multiple cytoplasmic branches towards the internal limiting basement membrane. The endings of these cytoplasmic branches underlie the internal

Figure 52. Major antigens detected by antiserum against crude frog pituitary glycoprotein extract. Immunoelectrophoresis of crude frog pituitary glycoprotein extract against anti-frog crude pituitary glycoprotein extract antiserum. Three precipitation arches are visible; the upper and middle arches moved towards the anode, the main portion of the lower arch remained at the origin. Electrophoresis was performed for 6 hr in 0.05 M barbital-acetate buffer pH 8.5, at 8.3 V/cm, 15 mA, at $5^{\circ}C$. The diagram at the bottom intends to clarify the photograph at the top.



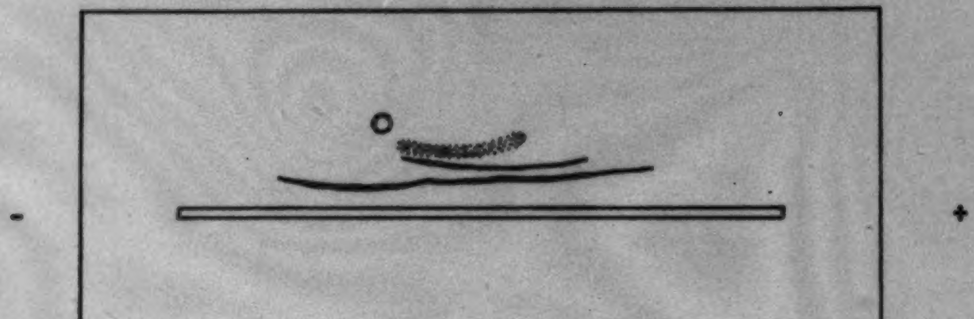
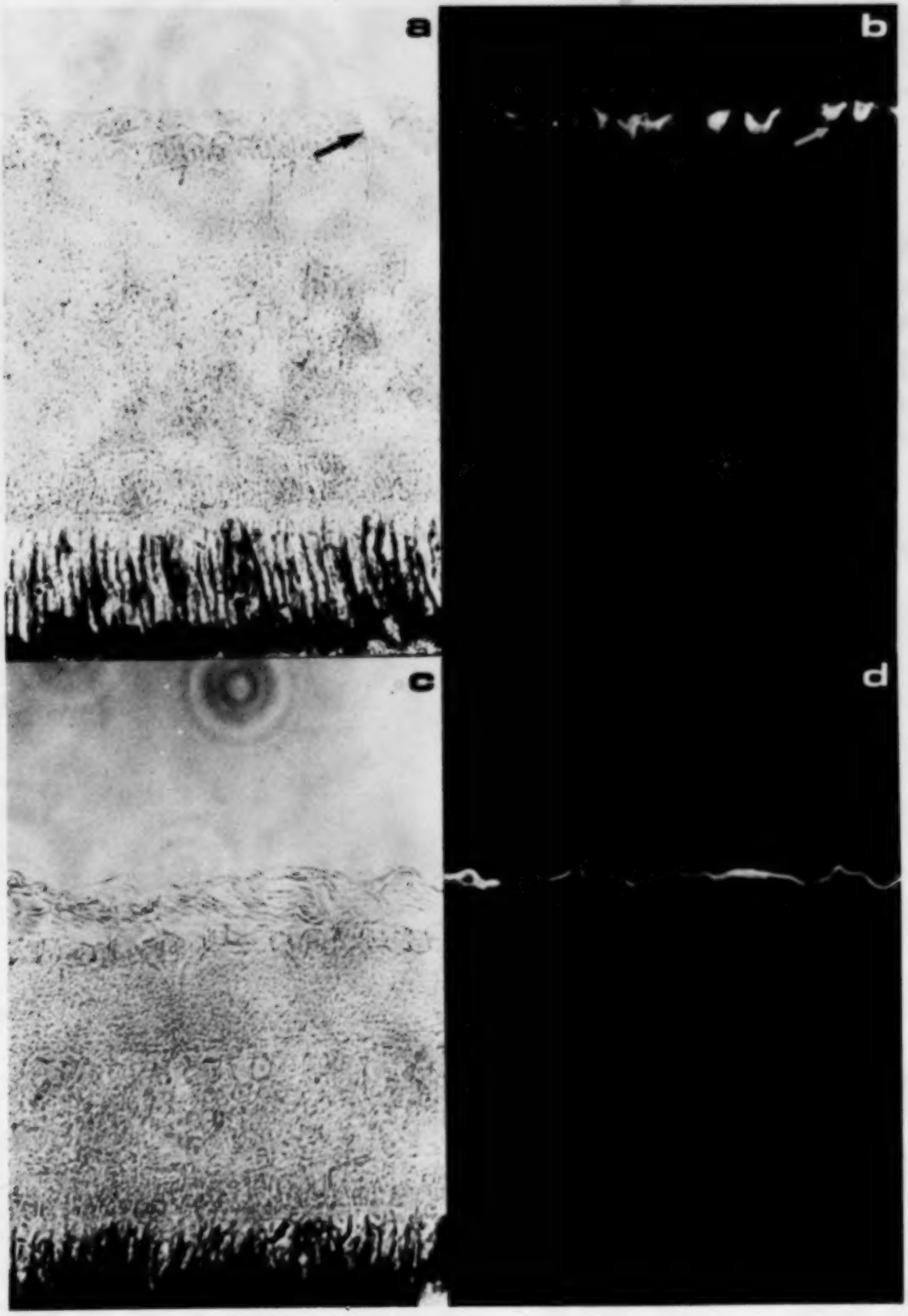


Figure 53. Replacement of inner limiting basement membrane by Müller's macroglial cells of the neural retina of the frog after lens removal; (a,b) 5 days after lentectomy, and (c,d) 14 days after lentectomy. (a,c) Unstained phase contrast light micrographs, and (b,d) corresponding immunofluorescent light micrographs. A rabbit antiserum against crude glycoprotein extract of frog (*Rana pipiens*) pituitary glands was used. The original inner limiting basement membrane is shed 5 days after lentectomy, but new material interacting with this antiserum is present in the internal portion of the Müller cells (arrow), at the level of the ganglion cell layer and nerve fiber layer. By 14 days a new inner limiting basement membrane has formed, but the fluorescent staining material in the Müller cells has disappeared. The fluorescence of the adult inner limiting membranes of the retina in nonlentecomized eyes is slightly weaker than in newly regenerated membranes. In other cell types of the neural retina only weak background staining was seen. (a,b) X 209, (c,d) X 197.


a
b
c
d

limiting basement membrane (Sarthy and Lam, 1978). Within 14 days after lentectomy, a new brightly fluorescent staining internal limiting basement membrane was present as examined in one frog eye. The cross-reacting substance in the internal processes of the Müller cells had disappeared by that time. This result suggests that the inner limiting membrane of the neural retina is shed after lentectomy. It is possible that this membrane is restored at least in part by the Müller cells. While the Müller cells synthesize molecules necessary for the replacement of the internal limiting basement membrane they could also produce a lens stimulating substance. However, it is also possible that the stimulating substance produced by the retina is different from the stimulating substance produced by the pituitary gland.

DISCUSSION

1. STIMULATORY EFFECTS OF VARIOUS HORMONE PREPARATIONS ON IRIS AND LENS

A variety of preparations of hormone-like substances has been reported to influence lens development and regeneration and to affect lens and iris cell differentiation in vertebrates. Lentropin, a protein extracted from embryonic chick vitreous body, promotes lens fiber differentiation in cultured lens epithelia (Beebe et al, 1980). This protein may be similar to the eye-derived growth factor from bovine neural retina (Arruti and Courtois, 1978), iris, pigment retina with choroid, and vitreous body (Barritault et al., 1981). Serum contains a protein of about 10^5 daltons molecular weight which can stimulate mitosis in most cell types (Holley and Kiernan, 1968, 1974); and also promotes lens fiber differentiation in lens epithelia of the chick embryo (Beebe et al., 1980).

Insulin is mitogenic for adult rabbit and other mammalian lens epithelial cells (Reddan et al., 1972, 1976, 1978); it also stimulates lens fiber differentiation in cultured embryonic chick lens epithelia (Piatigorsky, 1973; Milstone and Piatigorsky, 1977). Apparently it did not stimulate lens regeneration in the newt *in vivo* (Takano, 1962) or in organ culture (Connelly, 1977c, 1980) although it can substantially elevate the mitotic index (Cuny and Zalik, 1981). Although this hormone could stimulate some lens cell elongation in newt iridocorneal complexes cultured in serum-free medium, its effect was no greater than the effect of serum (Williams and McGlinn, 1979).

Fibroblast growth factors from bovine brain (Gospodarowicz et al.,

1978) and adenohypophysis (Gospodarowicz, 1975) stimulate proliferation of mouse 3T3 fibroblasts and the latter factor has been reported to increase the number of mitotic cells labelled with ^{3}H-thymidine in bovine lens epithelium and corneal epithelium (Gospodarowicz et al., 1977; Gospodarowicz and Ill, 1980). This factor, however, has no effect on human lens epithelial cells (Barritault et al., 1981). Epidermal growth factor from mouse submaxillary salivary glands (Cohen and Cohen, 1972) increased ^{3}H-thymidine labelling in cultured bovine lens epithelia, corneal epithelia, and corneal endothelia (Gospodarowicz and Greenburg, 1979). However, if cells of these tissues are dissociated this factor has no stimulatory effect (Gospodarowicz et al., 1977). Epidermal growth factor stimulated some cell elongation in iridocorneal complexes of the newt cultured in the absence of serum; this stimulatory effect was no greater than the effect of serum alone (Williams and McGlinn, 1979). A similar result was obtained when nerve growth factor (Cohen, 1960) from mouse salivary glands was used (Williams and McGlinn, 1979). This polypeptide promotes growth of adrenergic neurons (Harper et al., 1980). It may be of interest to mention that in the iris of the rabbit potassium-induced release of the neurotransmitter norepinephrine is higher in depigmented than in pigmented areas (Farah and Patil, 1979).

Prostaglandins mediate the inflammatory response in the rabbit iris (Cole and Unger, 1973; Eakins, 1977; Sakata and Yoshida, 1979). Injection of an antagonist of prostaglandin synthesis, indomethacin, does not prevent lens regeneration in the newt (Connelly, 1977a). Retinoic acid, a vitamin A derivative, slightly inhibits cell elongation and proliferation of bovine lens epithelial cells (Barritault et al., 1981).

Corticosteroids, such as dexamethasone, have been reported to stimulate elongation of cultured calf lens epithelial cells (Van Venrooji et al., 1974). Hydrocortisol injections did not accelerate lens regeneration in the newt (Takano, 1962), nor did cortisol stimulate this process in organ culture (Connelly, 1977c, 1980). Although a crude adrenocorticotropin preparation accelerated lens regeneration when injected intraperitoneally, this effect could not be reproduced in organ culture when the porcine adrenocorticotropin preparation ACTH-6002 was used (Figure 9). Since adrenocorticotropin stimulates production of corticosteroids in the cortex of the adrenal gland (Bloom and Fawcett, 1975), and corticosteroids are ineffective in the stimulation of lens regeneration, it is possible that the effect of adrenocorticotropin *in vivo* is either of a broader systemic nature, or could be due to a contaminant in the preparation used.

Mammalian prolactin does not stimulate lens regeneration in organ culture of newt iridocorneal complexes (Connelly, 1977c, 1980), or irises alone (Figure 10). On the other hand, crude frog prolactin injected intraperitoneally apparently stimulated mitosis in lens epithelia of hypophysectomized frogs (Wainwright et al., 1978).

Bovine somatotropin stimulates mitosis in lens epithelia of hypophysectomized frogs (*Rana pipines, R. catesbeiana*)(Rothstein et al., 1976; Van Buskirk et al., 1975) but not in the lens epithelia of lenses maintained in organ culture (Weinsieder and Roberts, 1980). This hormone stimulates production of somatomedins in the liver, which in turn act on various target tissues. Somatomedin C injected into hypophysectomized froglets (*Rana catesbeiana*) can stimulate mitosis in their lens epithelia

(Rothstein et al., 1979). In the present experiments bovine somatotropin preparation GH-B18 at high doses could stimulate a low frequency of lens regeneration from irises in organ culture (Figure 11). The fact that only high concentrations of this preparation had a stimulatory effect suggests that this effect may be due to a contaminant in the preparation.

Injection of sex steroids did not accelerate lens regeneration in the newt (Takano, 1962), but an ovine lutropin preparation has been reported to stimulate mitosis in lens epithelia of hypophysectomized frogs (*Rana pipiens, R. catesbeiana*)(Rothstein and Worgul, 1973; Van Buskirk et al., 1975). In the present experiments bovine lutropin LH-B10 did not stimulate lens regeneration (Figure 12). At a very high concentration the bovine follitropin preparation FSH-B1 had a weak stimulatory effect on lens regeneration in organ culture (Figure 13). Again this effect may be due to a contaminant in the preparation used.

Tri-and tetraiodothyronine, when injected intraperitoneally, stimulate mitotic activity in lens epithelia of hypophysectomized adult frogs (*Rana pipiens, R. catesbeiana*)(Rothstein and Worgul, 1973; Van Buskirk et al., 1975). These hormones, however, have no effect on cultured lens epithelia, (Weinsieder and Roberts, 1980), and tetraiodothyronine does not stimulate lens regeneration in the iris in organ culture (Connelly, 1977c, 1980). These thyroid hormones are hydrolytic products of the iodoprotein thyroglobulin, which is stored in colloid form in epithelial vesicles of the thyroid gland (Uhlenhut, 1927; Uhlenhut and Karns, 1928; Charipper, 1930; Grant, 1930; Herman, 1960; Dunn and Dent, 1980). Proliferation of thyroid epithelial cells (Isler, 1979; Nitsch and Wollman, 1980), thyroglobulin synthesis and its proteolytic cleavage (Tong, 1974; Dunn and Dent, 1980) are controlled by thyrotropin, a glycoprotein hormone secreted by thyrotropic cells (Dent, 1956, 1961; Dent and Gupta, 1967; Aplington, 1962; Doerr-Schott, 1966; Hauser-Gunsbourgh et al, 1973; Dunn and Dent, 1976; Olivereau et al., 1976, 1977; Schubert et al., 1977; Eagleson and McKeown, 1978) in the pars distalis of the pituitary gland (Atwell, 1921; Charipper, 1931; Roofe, 1937, 1938; Copeland, 1943; Kent, 1945; Miller and Robbins, 1955; Cardell, 1964a, b; Masur, 1969; Masur and Holtzman, 1969). Bovine thyrotropin injected into hypophysectomized frogs (*Rana pipiens, R. catesbeiana*) enhanced mitotic activity in lens epithelia (Rothstein and Worgul, 1973; Van Buskirk et al., 1975). When this hormone was injected into thyroidectomized frogs it had no effect (Weinsieder and Roberts, 1980). Also, the thyrotropin preparation Sigma TS-10 could not stimulate lens epithelial proliferation in cultured lenses. From this work, it appears that thyrotropin stimulates release of tri- and tetraiodothyronine, and these thyroid hormones probably increase the activity of an as yet unknown lens growth factor in the frog (Weinsieder and Roberts, 1980). In the present experiments some thyrotropin preparations could stimulate lens regeneration in organ culture (Figures 14, 48).

It is possible to explain the slightly stimulatory effects of high concentrations of follitropin FSH-B1 and somatotropin GH-B18 by contamination of these preparations by thyrotropin. FSH-B1 contains 0.02 I.U. TSH/mg and GH-B18 0.05 I.U. TSH/mg respectively (Reichert, 1977). Even though the thyrotropin activity was 0.17 I.U. TSH/mg in lutropin LH-B1 this preparation had a rather inhibitory effect on lens regeneration. LH-B10 may contain an inhibitor for lens regeneration in addition to thyrotropin, or thyrotropin may not be identical with the lens regeneration stimulating activity.

2. RELATIONSHIP OF THE LENS REGENERATION STIMULATING FACTOR FROM THE PITUITARY GLAND TO THYROTROPIN

One international unit (I.U.) of thyrotropin has been defined as the biological activity present in 20 mg of the bovine International Standard for Thyrotropin (Musset and Perry, 1955). Human, and also ovine, thyrotropin can validly be bioassayed against this standard preparation (Reichert, 1970). The purest preparations of bovine thyrotropin, which have been used for amino acid sequencing, have biological activities ranging from 30 to 90 I.U. TSH/mg (Pierce et al., 1973; Williams et al., 1980). The purest human thyrotropin preparations have reached activities of 20 I.U. TSH/mg (Condliffe and Robbins, 1966), and a better purification can now be achieved by immunoaffinity chromatography (Pekonen et al., 1980). Bovine thyrotropin TS-10 is the crudest preparation tested on lens regeneration (Figure 46), with a biological activity of about 0.75 I.U. TSH/mg (Sigma Chemical Comp., 1980) as determined by bioassay; this thyrotropin activity corresponds to approximately 1 to 2% thyrotropin by weight. This preparation had only a weak stimulatory effect. Only high concentrations of TS-10 (1400 μg/ml) significantly stimulated lens regeneration. The bovine thyrotropin preparation TSH-B8 has a biological activity of 3.5 I.U. TSH/mg (Reichert and Wilhelmi, 1978) as determined in the chicken, by a bioassay involving uptake of ^{32}P-phosphate by the thyroid. This corresponds to about 5 to 10% thyrotropin by weight. This preparation stimulated lens regeneration at concentrations as low as 3 μg/ml, corresponding to 0.01 I.U. TSH/ml (Figure 21). The ovine preparation of thyrotropin, oTSH-9, has an activity of 7.5 I.U. TSH/mg (Parlow, 1979), or 11 to 21% thyrotropin by weight. This preparation was highly active in stimulating lens regeneration at concentrations as low as 1.4 μg/ml, corresponding to 0.01 I.U. TSH/ml (Figure 48). These results suggest that the higher the degree of purification of the thyrotropin preparation, the higher its lens regeneration stimulating activity; this would be expected if thyrotropin was identical with the lens regeneration stimulating activity. However, probably the purest thyrotropin preparation tested was the human hTSH, which had an activity of 5 I.U. TSH/mg (CalBiochem Catalog, 1980), corresponding to approximately 13 to 25% thyrotropin by weight. This preparation did not stimulate lens regeneration at the only concentration tested, 1.6 μg/ml, or 0.008 I.U. TSH/ml (Figure 50). The activity of this preparation has been determined by radioimmunoassay, which may measure denatured thyrotropin in addition to active thyrotropin. If we assume that most of the thyrotropin in this preparation was biologically active, then the lens regeneration stimulating activity was presumably removed from this preparation and could be different from thyrotropin.

Evolutionary change of the amino acid sequence and carbohydrate composition may affect the biological activity in heterologous species (Pierce et al., 1973). Sheep and cattle are both ruminant artiodactyla, and the structure of their thyrotropin may be more similar than the one of man, a representative of catharrhina primates. Bovine thyrotropin seems to be able to stimulate the thyroid gland in the frog (Weinsieder and Roberts, 1980) but for human thyrotropin no information is available.

It may be mentioned, that an anuran bioassay has been developed, based on the rate of metamorphosis of frog tadpoles (*Rana pipiens, Xenopus laevis*), to measure thyrotropin activities from cattle, man, rat, sheep, horse, guinea pig and pigeon, and activities as low as 500 micro-Junkman-Schoeller units, equivalent to 50 μI.U. cattle thyrotropin are detectable (D'Angelo et al., 1942, 1950). The evidence given here cannot fully explain the total inactivity of the relatively pure human thyrotropin preparation, at a concentration of thyrotropin where other thyrotropin preparations have been active, because even thyrotropin with an altered structure could be expected to partially interact with the receptor molecules in the newt. Above all, the disparity of lens regeneration stimulating activities of the two bovine thyrotropin preparations TSH-B8 and TS-10 suggests that thyrotropin activity may be separable from the lens regeneration stimulating activity.

If thyrotropin itself is the lens regeneration stimulating factor in the thyrotropin preparations tested, then the same response of cultured dorsal irises should be expected with 3 μg/ml TSH-B8 (Figure 21), 1.4 μg/ml oTSH-9 (Figure 48), 14 μg/ml TS-10 (Figure 46), and 2 μg/ml hTSH (Figure 50), because the thyrotropin concentrations were the same, i.e. 0.01 I.U. TSH/ml. The concentration of thyrotropin in cultures with hTSH preparation was only 0.008 I.U. TSH/ml (Figure 50), but this should not abolish totally its stimulating effect. Indeed, very different responses have been obtained at this thyrotropin concentration. Ovine oTSH-9 and bovine TSH-B8 gave rise to advanced lens regenerates at significant frequency, but bovine TS-10 and human hTSH had no visible effect at all at this concentration. This result seems to suggest that the lens regeneration stimulating activity copurifies with thyrotropin only in some preparations, and that it may be an entity separable from thyrotropin.

Lens regeneration is a relatively complex process and is affected by many variables. Hence, it is not surprising that the individual lens regeneration responses of the cultured dorsal irises are variable, and the dose response curves are difficult to assess. When the variation of lens regeneration stages in control cultures is considered, the dose response of the different thyrotropin preparations can be compared; 3000 to 3 μg/ml TSH-B8 stimulated lens regeneration about equally well (Figures 14, 20, 21), although at 30 μg/ml a faint peak, and at 3 μg/ml a decline of the lens regeneration response could have occurred. At 0.3 μg/ml, the response dropped to an almost undiscernible level. The plateau region of the dose response of the oTSH-9 preparation extends down to at least 1.4 μg/ml. The absence of lens regeneration at 0.5 μg/ml is puzzling if one considers the strong response to this preparation when used at an only 3 times higher concentration. It is conceivable that in a larger experimental sample a weak response could be obtained. TS-10 can only stimulate at the very high dose of 1400 μg/ml, similar to the somatotropin GH-B18, and follitropin FSH-B1 preparations. The hTSH preparation could only be tested at one concentration and the dose response curve cannot be estimated. The lowest lens regeneration stimulating concentration of any of the thyrotropin preparations was 0.01 I.U. TSH/ml; this may be equivalent to 110 to 330 ng/ml thyrotropin. Basal levels of thyrotropin in normal human serum have been reported to be about 1.3 ng TSH/ml (Bigos et al., 1978), 0.84 to 1.65 ng TSH/ml

(Capri et al., 1979), or 0.6 to 2.8 ng TSH/ml, averaging at 1.8 ng TSH/ml (Odell et al., 1965); they can increase to 5.3 to 13.3 ng TSH/ml (Bigos et al., 1978), or 2.45 to 7.00 ng TSH/ml (Capri et al., 1979). Thyrotropin levels are high during the night, but low during the day (Wecke and Gandersen, 1978). Rats kept in total darkness also have an elevated serum thyrotropin level, but their level of tetraiodothyronine is lower than under light conditions (Ooka-Souda et al., 1977). During starvation, serum thyrotropin levels of young bulls decrease to 85% of the levels of well fed bulls, and this drop is paralleled by a decrease of serum tetraiodothyronine (Tveit and Almid, 1980). Pregnant women have a normal level of pituitary thyrotropin in their serum, 25 to 40 μI.U. TSH/ml (Harada et al., 1979), which may correspond to 0.3 to 2 ng/ml thyrotropin, although their placenta adds 0.25 μI.U./ml or 0.003 to 0.013 ng/ml of chorionic thyrotropin (Harada et al., 1979). In humans with hyperthyroidism serum levels of 63 to 149 ng/ml thyrotropin have been measured (Bigos et al., 1978), and 7 to 156 ng/ml thyrotropin in humans with primary myxedema (Odell et al., 1965). Therefore, the lowest concentration of thyrotropin, which stimulated lens regeneration was about 100 to 400 times higher than the basal normal thyrotropin level in human serum, 10 to 130 times higher than the elevated normal level, and about twice as high as the highest concentrations encountered under pathological conditions. Although the lowest lens regeneration stimulating concentration of thyrotropin preparations is still above physiological levels, it is reasonably close, especially when considering the very distant phylogenetic relationship between the mammalian source of the thyrotropin and the target tissue, the newt iris. This finding suggests the possibility that thyrotropin itself could stimulate lens regeneration.

Cultured lens regenerates, compared to those *in vivo*, show different degrees of abnormalities. Morphological aberrations have also been found in lens regenerates arising from irises cultured with pituitary glands (Connelly et al., 1973) and larval frog retinas (Yamada et al., 1973), and can also be observed *in vivo* in lenses regenerating from iris grafts in the eye (Wachs, 1914; Reyer, 1956). These morphological deficiencies can be expected in the culture situation where the natural morphogenetic fields cannot be correctly stimulated.

Regeneration of a lens from the iris is dependent on mitotic activity (Donaldson, 1972; Yamada, 1977) and hence lens regeneration stimulating agents should be able to elevate the mitotic rate within the iris epithelial cell population. The thyrotropin preparation TSH-B8 can stimulate mitosis in cultured newt irises (Figure 17); therefore this preparation appears to stimulate lens regeneration via the normal sequence of events involving cell division. Highly purified thyrotropin preparations have also been reported to stimulate mitosis in thyroid epithelial cells (Nitsch and Wollman, 1980).

The presence of γ-crystallin has been used as an indication of lens fiber differentiation in organ culture (Yamada et al., 1973; Connelly, 1977b). During this study, γ-crystallin was detected by immunofluorescence in all fiber cells which could be recognized histologically and by their eosinophilic staining reaction. As in the *in vivo* situation (McDevitt and Brahma, 1979), γ-crystallin usually appears at the late lens vesicle stage 5 on cultured dorsal irises. In the presence of either pituitary gland, TSH-B8, or oTSH-9, some cultured irises, in which a regenerated lens was not visible, contained a population of depigmented cells, sometimes scattered within the iris, which were positive for γ-crystallin. This suggests that even in the absence of a morphologically distinguishable lens some cell type transformation could have occurred. Neither in control cultures, nor in cultures supplemented with thyrotropin preparations TS-10 or hTSH could γ-crystallin be found; this is in agreement with the findings based on the morphological staging system.

Summarizing, it may be stated that although thyrotropin TSH-B8 and oTSH-9 are the only anterior pituitary hormone preparations tested which can stimulate lens regeneration at relatively low concentrations the relative inactivity of thyrotropin TS-10 and hTSH at comparable concentrations cannot satisfactorily be explained by evolutionary molecular modifications, impurities, or inadequacy of assay methods. It is probable that it will be possible to separate the thyrotropin activity from the lens regeneration stimulating activity in pituitary hormone preparations by the use of physicochemical or immunoaffinity methods.

3. PREDICTIONS FOR THE PHYSICOCHEMICAL PROPERTIES OF THE LENS REGENERATION STIMULATING FACTOR

From the organ culture experiments it appears that the lens regeneration stimulating activity copurifies, to a certain degree, with thyrotropin TSH-B8 and oTSH-9, and to a lesser extent with follitropin FSH-B1, somatotropin GH-B18, and thyrotropin TS-10. The methods of purification of these preparations have been published, and summaries of the steps followed in the preparation of these thyrotropins are given in Figures 54 to 57. From the lens regeneration stimulating potencies of these preparations, obtained by different fractionation procedures, some physicochemical properties of the lens regeneration stimulating factor in the pituitary gland can be predicted.

Bovine thyrotropin, as well as thyrotropin of other vertebrates, is a glycoprotein. Its molecular weight is 28.3 x 10^3 daltons (Pierce et al., 1973), and it has an isoelectric point near pH 7.0 (Papkoff, 1972). It is composed of an α-subunit of 13.6 x 10^3 daltons and a β-subunit of 14.7 x 10^3 daltons (Pierce et al., 1973), and these two subunits are translated on two independent cistrons (Kourides and Weintraub, 1979). The α-subunits of follitropin and lutropin are identical with the α-subunit of thyrotropin, but the β-subunit is specific for thyrotropin (Papkoff, 1972), although its amino acid sequence (Liao and Pierce, 1971) shows a 50% homology with the β-subunit of lutropin (Pierce et al., 1973). Thyrotropin consists of 85.8% peptide and 14.2% carbohydrate (Howard and Pierce, 1969). The secreted bovine α-subunit of thyrotropin has 96 amino acid residues and 2 carbohydrate chains, which account for 20.6% of the molecular weight of this subunit, and are linked to asparagine (Pierce et al., 1973). The β-subunit has 113 amino acid residues and only 1 carbohydrate chain, the latter accounting for 9.9% of its weight, and also linked to asparagine (Pierce et al., 1973). In contrast to bovine thyrotropin, human thyrotropin contains sialic acid (Pierce et al., 1973; Hara et al., 1978). Human thyrotropin also has a molecular weight of about 28 x 10^3 daltons (Condliffe and Robbins, 1966); 21% of the weight is contributed by the three carbohydrate chains, whereby the α-subunit carries double the amount of the β-subunit (Pierce et al., 1973; Hara et al., 1978). Thyrotropin has also been isolated from bullfrog pituitary glands (McKenzie and Licht, 1978), but its molecular structure is not known as yet.

Like thyrotropin, the lens regeneration stimulating factor is probably a macromolecule larger than 8 x 10^3 or 12 x 10^3 daltons, because it is nondialyzable. Dialysis has been used during the purification of all thyrotropin preparations (Figures 54 to 57) used here. The lens regeneration stimulating factor seems to have a molecular weight different from thyrotropin, because the human thyrotropin preparation hTSH did not contain lens regeneration stimulating activity. This is the only preparation which has been subjected to molecular weight fractionation by gel-filtration chromatography on Sephadex G-100 (Figure 57). Thyrotropin elutes in the second peak (Stockell Hartree, 1975), and the first higher molecular weight peak could in theory contain the lens regeneration stimulating activity. The lens regeneration stimulating factor could be linked to molecules ranging in weight from 8 x 10^3 to about 25 x 10^3 or larger than 30 x 10^3 daltons.

The lens regeneration stimulating factor is probably not lipophilic, because it has not been extracted with ethyl ether or absolute acetone during the preparation of oTSH-9 (Figure 56). However, there is an unlikely possibility that it was precipitated in 1:1 water-acetone or extracted in 1:2 water-acetone mixtures, conditions used to prepare slightly active TS-10 powder (Figure 55). The lens regeneration stimulating factor seems to be relatively resistant to repeated freezing

Figure 54. Flow chart of the purification procedure for bovine pituitary thyrotropin preparation NIH-TSH-B8 according to Reichert (1975). The biological activity of the final product as determined by bioassay in the chicken thyroid involving the uptake of ^{32}P-phosphate was 3.5 I.U. TSH/mg (Reichert and Wilhelmi, 1978).

bovine pituitary glands: frozen, ground

▼

extraction: distilled water, pH 5.5, 16 hr → FSH

▼

extraction of residue: ammonium sulfate,
0.1 M, pH 4.0 → PRL + GH

▼

precipitation from supernatant: ammonium sulfate,
1.4 M, pH 4.0, 16 hr

▼

precipitation from supernatant: ammonium sulfate,
3.5 M, pH 4.0, 16 hr

▼

ion exchange chromatography of dialyzed supernatant:
weakly acidic carboxylic polymethacrylate
adsorbed: 0.007 M phosphate - 0.003 M
borate buffer, pH 8.0, 1 hr
eluted: ammonium sulfate, 0.5 M, or sodium
chloride, 1.0 M in buffer, pH 8

▼

precipitation of eluted fraction: ammonium sulfate,
3.0 M, pH 5.5

▼

ion exchange chromatography of dialyzed precipitate:
diethylaminoethylcellulose
adsorbed: 0.005 M glycine buffer, pH 9.5
eluted: sodium chloride, 1.0 M in buffer, → LH
pH 9.5

▼

peroxidation of dialyzed eluted fraction:
hydrogen peroxide, 1 M, in distilled
water, pH 5.5, 22°C

▼

dialysis: 3°C, 2 days, extensive, lyophilized. → TSH

Figure 55. Flow chart of the purification procedure for bovine pituitary thyrotropin preparation Sigma-TS-10 according to Ciereszko (1945). The biological activity of the final product in an unidentified bioassay was 0.75 I.U. TSH/mg (Sigma Chemical Comp., 1980).

bovine pituitary glands: frozen, ground

▼

extraction: sodium chloride, 0.34 M, pH 7.4 to 7.8, 3 hr

▼

precipitation from supernatant: sodium chloride, 0.34 M
pH 4.0 to 4.1

▼

precipitation from supernatant: acetone added 1:1, 16 hr

▼

precipitation from supernatant: acetone added 1:2, 16 hr

▼

washing of this last precipitate: acetone-water 3:1,
3 times

▼

drying of precipitate: pure acetone, followed by
ethyl ether

▼

extraction of powder: distilled water, pH 9.0

▼

precipitation from supernatant: lead acetate, 0.154 M,
pH 5.0 to 5.5, 1 hr

▼

precipitation from supernatant: trichloroacetate,
final concentration 0.5 M, pH 1.2

▼

dialysis: running tap water, lyophilized → TSH

Figure 56. Flow chart of the purification procedure for ovine pituitary thyrotropin preparation NIAMDD-oTSH-9 according to Parlow (1979). The biological activity of the final product measured by a bioassay involving ^{131}I-iodine increase in the mouse serum was 7.5 I.U. TSH/mg (Parlow, 1979).

ovine pituitary glands: acetone, ground

▼

extraction: 10% ammonium acetate, pH 5.1 (acetic acid)
96% ethanol mixed 3:2, 24 hr → PRL + GH

▼

precipitation: ethanol added 1:2, 24 hr

▼

ion exchange chromatography of dialyzed precipitate:
carboxymethylcellulose
adsorbed: ammonium acetate, 0.005 M, pH 5.5
→ FSH
eluted: ammonium acetate, 1.0 M, pH 5.5

▼

ion exchange chromatography of dialyzed eluted fraction:
diethylaminoethylcellulose
adsorbed: glycine, 0.005 M, pH 9.5, glycine
concentration increased → LH
eluted: glycine, 0.2 M, pH 9.5

▼

ion exchange chromatography of dialyzed eluted fraction:
diethylaminoethylcellulose
adsorbed and eluted as before

▼

dialysis: distilled water, lyophilized → TSH

Figure 57. Flow chart of the purification procedure for human pituitary thyrotropin preparation CalBiochem-hTSH according to Kritchevsky (1981, personal communication). The immunochemical activity of the final product in a radioimmunoassay corresponded to 5.0 I.U. TSH/mg. Apparently, the method of purification introduced by Stockell Hartree (1975) was closely followed, with the omission of the final ion exchange chromatography step on DE-32.

human pituitary glands: acetone, ground

▼

extraction: 10% ammonium acetate, pH 5.1 (acetic acid)
96% ethanol mixed 3:2, 24 hr → PRL + GH

▼

precipitation: ethanol added 1:2, 24 hr

▼

ion exchange chromatography of dialyzed precipitate:
probably: carboxymethylcellulose
adsorbed: ammonium acetate 0.004 M, pH 5.5
→ FSH
eluted: ammonium acetate, 1.0 M, pH 5.5

▼

ion exchange chromatography of dialyzed eluted fraction:
probably: diethylaminoethylcellulose DE 23
adsorbed: glycine, 0.1 M, pH 9.5 → LH
eluted: ammonium acetate, 1.0 M, pH 5.5

▼

ion exchange chromatography of dialyzed eluted fraction:
probably: Amberlite IRC-50
adsorbed: phosphate buffer, pH 7.5, containing
0.005 M sodium ion
eluted: ammonium acetate, 1.0 M, pH 5.5

▼

precipitation of eluted fraction: ethanol added 1:5,
48 hr; precipitate washed with ethanol
and ether and dried

▼

gel filtration chromatography of precipitate dissolved
in glycine buffer:
Sephadex G-100
eluted: glycine, 0.1 M, pH 9.5, 2nd peak collected

▼

dialysis: distilled water, lyophilized → TSH and thawing in aqueous solutions, since it has been extracted from frozen glands to prepare TSH-B8 and TS-10, and has been stored frozen in culture medium before use. This factor is soluble in mixtures of 40% ethanol-6% aqueous ammonium acetate, but is insoluble in mixtures of 80% ethanol-2% aqueous ammonium acetate, which have been used during the preparation of oTSH-9. Prolactin PRL-B4 has been extracted with 75% ethanol-water mixtures, in which the lens regeneration stimulating factor was most likely insoluble. This can well explain the inactivity of the PRL-B4 preparation.

The lens regeneration stimulating factor could be partially soluble in distilled water at pH 5.5, because the slightly active FSH-B1 preparation which presumably has this factor as a contaminant has initially been extracted under this condition (Figure 54). Thyrotropin TS-10 has been dissolved in distilled water at pH 9 (Figure 55), and there is an unlikely chance that the lens regeneration stimulating factor does not solubilize at this pH. On the other hand, the somatotropin preparation GH-B18 has been precipitated in distilled water at pH 5.3, and some lens regeneration stimulating activity seems to have coprecipitated with this preparation. Hence, the isoelectric point of this factor may be close to that pH. Lens regeneration stimulating activity could have been absorbed to insoluble weak or moderately strong cation exchangers, such as carboxymethyl polymethacrylate at pH 8.0 during the preparation of TSH-B8 (Figure 54), or carboxymethylcellulose at pH 5.5 during the preparation of oTSH-9 (Figure 56), and could probably also have been adsorbed to the Amberlite resin IRC-50 at pH 7.5 during the preparation of hTSH (Figure 57). At pH 9.5, this factor seems to adsorb to weak anion exchangers, such as diethylaminoethylcellulose, like thyrotropin in the TSH-B8 and oTSH-9 preparations (Figures 54, 56).

Contaminating lutropin activity in the TSH-B8 preparation could be diminished by oxidation of the glycoproteins in 1 M hydrogen peroxide for 1 hr (Reichert, 1969, 1975), a condition which affected the thyrotropin activity only slightly. In addition, the lens regeneration stimulating factor is still biologically active after this treatment, suggesting that it is relatively oxidation resistant. Lipid-like compounds would have been oxidized during this treatment, which argues against a lipid-like nature of this factor.

The lens regeneration stimulating factor can be precipitated in 3.5 M ammonium sulfate at pH 4.0, or 3.0 M ammonium sulfate at pH 5.5, as has been used during the preparation of TSH-B8 (Figure 54). It may be only partially insoluble in 2.0 M ammonium sulfate at pH 7.0, because some lens regeneration stimulating activity has partially coprecipitated with somatotropin GH-B18 under this condition. Only precipitation methods have been applied to extract the GH-B18 preparation, and this preparation could have been contaminated with various pituitary peptides. On the other hand, the lens regeneration stimulating factor seems to be soluble at lower salt concentrations used in culture medium, as well as in solutions used in the extraction steps mentioned in Figures 54 to 57. Some lens regeneration stimulating activity could have been precipitated with TS-10 in 0.154 M lead acetate at pH 5.0 to 5.5.

In view of the difficulty of separation of bovine lutropin (Figure 12) and thyrotropin (Figure 13) activities by physicochemical methods, it is striking that lutropin LH-B10 had no stimulatory effect on lens regeneration, and was rather inhibitory, while thyrotropin TSH-B8 was highly stimulatory. Oxidation with hydrogen peroxide may have destroyed an inhibitor in the thyrotropin preparation, which remained active in the untreated lutropin preparation. It is less likely that anion exchange chromatography (Figure 54) could have completely separated this factor from the LH-B10 fraction together with the TSH-B8 fraction. The TS-10 preparation may have contained toxic chemicals remaining from the isolation procedure, such as lead acetate and trichloroacetic acid. In fact, when dissolving large amounts of TS-10 in culture medium, the indicator dye phenol red indicated a drop in pH, which needed to be readjusted. However, the most likely reason for the failure of TS-10 to stimulate lens regeneration is probably the exposure to pH 1.2 in trichloroacetic acid, a pH which does not hydrolize thyrotropin (Ciereszko, 1945), but may affect the lens regeneration stimulating factor.

As has been outlined above, some chemical properties of the lens regeneration stimulating factor can be predicted from the purification procedures used in the various hormone preparations in which it is probably present as a contaminant. This factor seems to be a macromolecule ranging in molecular weight either from 8 x 10^3 to 25 x 10^3, or higher than 30 x 10^3 daltons, and is probably a peptide with an isoelectric point in the region of pH 5. Its resistance to oxidation and its insolubility in acetone support the idea that it may not be related to lipids. It may be partially hydrolysed at pH 1.2, but is probably resistant to this process at pH 4.0.

4. RELATIONSHIP OF THE LENS REGENERATION STIMULATING FACTOR TO LENTROPIN OR PITUITARY FIBROBLAST GROWTH FACTOR

With the information presented above it is now interesting to compare the properties of chicken vitreous lentropin (Beebe et al., 1980), the bovine eye-derived growth factor (Barritault et al., 1981) and the bovine pituitary fibroblast growth factor (Gospodarowicz, 1975) with the predicted properties of the lens regeneration stimulating factor. Because only small amounts of lentropin could be isolated by the above mentioned workers, the degree of purity was not very high, and the characterization of this protein is still incomplete (Figure 58). Lentropin was unstable during gel-filtration in the absence of horse plasma, probably due to the presence of proteolytic enzymes, since lentropin was destroyed by trypsin (Beebe et al., 1980). Its molecular weight of 60 x 10^3 daltons is compatible with the notion that it may be similar to the lens regeneration stimulating activity from bovine pituitary glands.

Barritault et al. (1981) have further characterized the stimulating factor present in neutral aqueous extracts of bovine retinas (Arruti and Courtois, 1978). The activity is linked to molecular weights larger than 50 x 10^3 daltons and an isoelectric point of pH 4.5 to 5. Urea (8 M) did not decrease the biological activity, suggesting that this factor has only one subunit. These properties are very similar to the ones of lentropin (Beebe et al., 1980). This extract can be cytotoxic for bovine, human, or mouse 3T3 fibroblasts. Similar activities could also be extracted from bovine iris, pigment epithelium with choroid and vitreous body. The ubiquitous origin of this eye-derived growth

Figure 58. Flow chart of the purification procedure for the embryonic chick lentropin preparation, according to Beebe et al. (1980). The biological activity of the final product was measured by a bioassay involving lens epithelial cell elongation in embryonic chick lenses (Beebe et al., 1980).

chick embryo vitreous bodies: fresh

▼

separation of vitreous humour: centrifugation at
26000 g, 4°C, 10 min

▼

centrifugal gel filtration of vitreous humour: desalting.
Sephadex G-25
eluted: distilled water, pH 6.1

▼

gel filtration chromatography of eluted fraction:
Sephadex G-75 or G-100 or Ultrogel AcA 34,
eluted: Ham's F-10 culture medium ± 2%
horse plasma → lentropin

factor casts a doubt on its identity with the lens regeneration stimulating factor, because in the newt eye, only the neural retina has been known to stimulate lens regeneration (Reyer, 1977a; Yamada, 1977), and iris cultured by itself remains largely pigmented (Eguchi, 1967).

Bovine pituitary fibroblast growth factor (Figure 59) can stimulate mitosis in lens epithelia (Gospodarowicz et al., 1977), and it is known to contaminate pituitary hormone preparations, especially the glycoprotein hormone preparations NIH-LH-B7 and NIH-TSH-B6 (Gospodarowicz et al., 1975). Lutropin NIH-LH-B10 did not stimulate lens regeneration, which argues against the possibility that the lens regeneration stimulating factor is identical with the pituitary fibroblast growth factor. Fibroblast growth factor can be separated from the glycoprotein hormones by gel filtration chromatography, because it has a smaller molecular weight of only 13.3×10^3 daltons (Gospodarowicz, 1975). This factor has also been shown to stimulate proliferation of rabbit articular chondrocytes (Barritault et al., 1981) and frog chondrocytes in amputated hind limb stumps (Gospodarowicz et al., 1978). Mitotic divisions in cultured rabbit articular chondrocytes were also enhanced by high concentrations of bovine thyrotropin preparations TSH-B5 or B6, at the lowest 69.5 μg/ml (Malemud and Sokoloff, 1974). Corvol et al. (1972) reported that many of the crude thyrotropin as well as other pituitary hormone preparations stimulated the incorporation of ^{3}H-thymidine in these cells. The lowest effective concentration of thyrotropin NIH-TSH-B5 was 1 μg/ml or 0.003 I.U. TSH/ml. It is possible that the chondrocyte stimulating activity corresponds to the mouse 3T3 fibroblast growth-promoting activity observed in thyrotropin and

Figure 59. Flow chart of the purification procedure for bovine pituitary fibroblast growth factor preparation CR-FGF-40002 according to Gospodarowicz (1975) as modified by Gospodarowicz et al. (1978). The biological activity of the final product as determined by the increase in the uptake of ^{3}H-thymidine in mouse 3T3 fibroblasts was probably about 2500 lutropin NIH-LH-B7-U/mg (Gospodarowicz, 1975).

bovine pituitary glands: frozen fresh, ground

▼

extraction: ammonium sulfate, 0.15 M, pH 7.0, 2 hr

▼

precipitation from supernatant: metaphosphate, 0.5 M,
pH 3.5

▼

precipitation from supernatant: ammonium sulfate, 2.2 M,
pH 6.5 to 7.0 (1 M sodium hydroxide)

▼

precipitation from supernatant: ammonium sulfate, 4.1 M,
pH 7.0

▼

ion exchange chromatography of dialyzed final precipitate:
carboxymethylsephadex C-50
adsorbed: sodium phosphate, 0.1 M, pH 6.0
eluted: sodium chloride, 1.0 M, in buffer

▼

gel filtration chromatography of dialyzed eluted fraction:
Sephadex G-75
eluted: ammonium carbonate, 0.1 M, pH 8.5,
3rd peak collected

▼

gel filtration chromatography of lyophilized eluted fraction:
Sephadex G-50
eluted: ammonium carbonate, 0.1 M, pH 8.5,
central part of single peak collected

▼

ion exchange chromatography of lyophilized eluted fraction:
carboxymethylcellulose CM-52
adsorbed: ammonium formate, 0.2 M, pH 6.0
eluted: ammonium formate, gradient 0.2 M to
0.4 M, 2nd peak collected

▼

lyophilized directly → FGF

chorionic gonadotropin preparations (Holley and Kiernan, 1968). In the present experiments pituitary fibroblast growth factor FGF-40002 did not stimulate lens regeneration. Because of the observed cytotoxicity of this preparation (Figure 51), fibroblast growth factor could be responsible for the inhibitory action found in the preparations TS-10, LH-B10, or less probable PRL-B4.

It is concluded that the lens regeneration stimulating factor in the bovine pituitary gland is most likely different from pituitary fibroblast growth factor and pituitary thyrotropin, but it could be similar to lentropin or eye-derived growth factor.

5. THOUGHTS ON THE MODE OF ACTION OF THE LENS REGENERATION STIMULATING FACTOR ON IRIS EPITHELIAL CELLS IN CELL CULTURE

Although a few of the dissociated iris cells had attached after two hours in culture, most of them required several days to accomplish this process. By 10 days 19 to 60% of the cells had attached; this percentage seems to be even higher with *Cynops pyrrhogaster* cells (Abe and Eguchi, 1977). Many cells however, remained spherical, and although they usually stuck to the substratum for a few days, they detached later on. These cells probably have been damaged severely during dissociation. The presence of TSH-B8 did not seem to appreciably stimulate attachment, and in general spreading was slightly inhibited.

In the early period of culture, the lag phase, dorsal iris epithelial cells remained stationary and spread out to attain a disc shape, also called lamellar configuration (Yamada, 1977). Some of them assumed a polarized spindle shape and migrated at various speeds across the dish (Abe and Eguchi, 1977; Yamada, 1977; Yamada and McDevitt, 1981).

In the newt eye iris epithelial cells, in addition to shielding the retina from light, probably act as nonspecialized sphincter muscles in a similar fashion as in the frog (Nolte and Pointner, 1975). Hence, these cells are well equipped for contraction and locomotion in cell culture. After they have transformed into γ-crystallin containing cells, they are at first very mobile (Figure 25 c,d), but possibly become nonmotile during terminal differentiation. No attention has been paid yet to the influence of TSH-B8 on motility of iris epithelial cells.

Highly pigmented iris epithelial cells appear less pigmented when they are spread out, especially at the cell periphery and above the nucleus. After a few days in culture, many of these cells (Figure 25 a,b) begin to actively shed cytoplasm and extrude melanin granules (Horstman and Zalik, 1974). The present observations indicate that depigmentation (Figure 30 a,b) is not stimulated by TSH-B8, since no differences in frequency, speed or degree of depigmentation were noticed between control and TSH-B8 supplemented cultures. During the logarithmic proliferation phase, melanin is either not resynthesized in sufficient amounts or is produced with a considerable delay, so that after each mitotic division the pigmentation becomes more and more diluted (Abe and Eguchi, 1977; Yamada and McDevitt, 1981). There is proportionally less depigmentation in cell populations at high density, probably because cell proliferation is down-regulated at saturation cell density (Holley, 1975). In *Cynops pyrrhogaster* this occurs at a population density of about 30×10^3 to 40×10^3 iris epithelial cells per cm^2 (Abe and Eguchi, 1977). Saturation cell density is reached before confluence of the cells (Holley, 1975). Under these conditions, dedifferentiation may be hampered by accumulation of extracellular matrix material produced by the cells. Collagen has been shown to inhibit cell type conversion of chick embryonic retinal epithelial cells in cell culture (Eguchi, 1979).

The onset of mitotic cell divisions in cultured iris epithelial cells is not accelerated by TSH-B8, because cells in control cultures begin dividing at the same time. The first mitotic figures were noticed after 10 to 15 days of culture in control and TSH-B8-supplemented cultures (Figures 36, 39, 42, 27, 28, 29). In the intact eye, iris epithelial cells are arrested in the diploid state, termed gap 0, or G_0 (Yamada, 1977). During activation, after lentectomy they progress into gap 1 or G_1, and reenter the cell cycle. In a population of irises there is considerable variability (Yamada, 1977; Eisenberg-Zalik and Yamada, 1967; Mitashov, 1969b), due to different genetic background, metabolic conditions linked to feeding, stress, age, season, temperature, etc. Iris epithelial cells have to pass through at least 6 mitotic cell cycles to convert into lens cells (Yamada, 1977; Yamada and McDevitt, 1981). Apparently 7 to 9 such cycles occurred in clonal cell cultures before lentoid bodies formed (Abe and Eguchi, 1977). Yamada and Beauchamp (1978) have suggested that the retinal lens regeneration stimulating factor elicits lens regeneration by shortening the duration of the cell cycle in dorsal iris melanocytes. However, the cell cycle of iris epithelial cells in primary cell culture was 1.88 times longer than in the eye and lentoid bodies still appeared (Yamada and Beauchamp, 1978). Also, ventral iris epithelial cells, which also produce lentoid bodies in cell culture, do not regenerate a lens *in vivo* (Eguchi et al., 1974). They had the same cell cycle time in cell culture as dorsal iris epithelial cells (Yamada and Beauchamp, 1978). The authors speculated that in the eye, the cell cycle time of ventral iris epithelial cells was much longer. Horstman and Zalik (1974) found that the cell cycle time of dorsal iris epithelial cells in cell culture was similar to the one in the iris in the eye, 67 hours. Iris epithelial cells did not seem to enter mitosis at an earlier time when stimulated with TSH-B8; the frequency of mitotic cells, however, was elevated by TSH-B8, especially in the first mitotic period. Although the above mentioned evidence is not sufficient to confirm or rule out that lens regeneration stimulating factors can accelerate the cell cycle, another mode of control may be proposed, based on evidence which has emerged in another system.

In the embryonic 3T3 mouse fibroblast cell line lag phases 1 and 2 can be distinguished, probably corresponding to G_0 and G_1. In cultures that have reached saturation density these cells have been arrested in G_0 (Holley, 1975) similar to the situation with the iris epithelial cells. Upon subculturing at low cell density, lag 1, a hormone independent phase, must be passed first (Jimenez de Asua et al., 1977). The duration of lag 2, however, can be shortened slightly by prostaglandin $F_{2\alpha}$ (Jimenez de Asua et al., 1977), or bovine pituitary fibroblast growth factor (Richmond et al., 1980). Insulin potentiates the action of both stimulators (Richmond et al., 1980), but has no effect on the duration of lag 2 when given alone (Jimenez de Asua et al., 1977). These cells must undergo an ordered sequence of cellular activation events to progress from G_1 to a threshold beyond which they become committed to deoxyribonucleic acid (DNA) synthesis (Pledger et al., 1978). In a population of dorsal iris epithelial cells TSH-B8 could increase mitosis in some responsive cells by lowering the threshold for the transition from the G_1 to the S or synthesis phase, and ultimately by recruiting more cells into the cell cycle.

At this moment it is not clear why the frequency of mitotic cells may reach several peaks during cultivation. Two peaks are, however, also observed during lens regeneration in the eye (Yamada and Roesel, 1969; Eguchi and Shingai, 1971). The peaks did not correlate with the change of culture medium every 7 days, nor with increased amounts of TSH-B8 after several additions, since controls showed similar fluctuation patterns (Figure 27, 28, 29).

Several peptide hormones, including thyrotropin (Williams et al., 1980), are known to stimulate adenylate cyclase activity in the plasmalemma of their target cells during specific interaction with receptors on the cell surface. During initiation of lens regeneration, the endogenous level of adenosine 3':5'-cyclic monophosphate is significantly reduced; it reaches its lowest point around day 2 after lentectomy (Thorpe et al., 1974), and increases markedly at days 3 to 18 after lentectomy (Velázquez and Ortíz, 1980). The present results indicate that the presence of TSH-B8 is not necessary during the first 4 days of cell culture, and iris epithelial cells may not be responsive to this preparation during early stages of activation. In later periods of cell culture TSH-B8 is necessary for elevated mitotic activity, suggesting that this preparation is necessary for redifferentiation, and not dedifferentiation. Thorpe et al. (1974) noticed that dedifferentiation was negatively correlated with endogenous adenosine

3':5'-cyclic monophosphate levels, but Yamada (1976) nevertheless postulated that this compound may stimulate melanin granule discharge during dedifferentiation.

Just before mitosis, most iris epithelial cells detach from their substrate to various degrees, a state termed stellate configuration (Ortíz et al., 1973; Yamada, 1977), as opposed to the lamellar configuration (Yamada, 1977) of the nonmitotic cell. Ortíz et al. (1973) could reversibly convert some quiescent iris epithelial cells in a lamellar configuration into the stellate form by microinjection of 0.01 mM adenosine, 0.01 mM 5'-adenosine monophosphate, 5.0 mM adenosine 3':5'-cyclic monophosphate and 1.0 mM dibutyryl or monobutyryl adenosine 3':5'-cyclic monophosphate (Yamada, 1977). This effect was abolished when 1.5 mM dibutyryl guanosine 3':5'-cyclic monophosphate was simultaneously applied with phosphorylated adenosine derivatives (Yamada, 1977). This suggests that phosphorylated guanosine derivatives may be antagonists of the phosphorylated adenosine derivatives in the control of cell shape (Yamada, 1977). It is probable that a protein phosphokinase, which catalyses phosphopeptide linkage formation on proteins using adenosine triphosphate as substrate, is involved in this change of cell shape, because these enzymes are known to depend on adenosine 3':5'-cyclic monophosphate. The latter compound is formed from adenosine triphosphate by the cell membrane-bound enzyme adenylate cyclase and is broken down by phosphodiesterases. Theophylline or papaverine, which are inhibitors of phosphodiesterase activity, can also cause stellate configuration in iris epithelial cells in culture (Ortíz and Yamada, 1975; Yamada, 1977). It can be assumed that while phosphodiesterase activity is blocked, endogenously produced adenosine 3':5'-cyclic monophosphate will accumulate through the activity of adenylate cyclase (Ortíz and Yamada, 1975; Yamada, 1977).

Adenosine 3':5'-cyclic monophosphate stimulated stellate transformation of lamellar iris epithelial cells is prevented (Ortíz and Connelly, 1977), and *in vivo* lens regeneration is disturbed or arrested after colchicine treatment (Hornsby and Zalik, 1977). Colchicine disrupts the microtubules, cytoskeletal elements which are indispensable for cytokinesis during mitotic divisions, a prerequisite for lens regeneration. Microtubules may also be involved in lens fiber cell elongation (Hornsby and Zalik, 1977), although chick embryonic lens epithelial cells can swell and elongate in the absence of polymerized microtubules (Beebe et al., 1979). Microtubules may be necessary for active retraction of cell processes during the acquisition of a stellate configuration (Ortíz and Connelly, 1977; Dumont and Yamada, 1972). Polymerization of tubulin to microtubules is dependent on phosphorylation of tubulin by an adenosine 3':5'-cyclic monophosphate-controlled protein phosphokinase (DeRobertis and DeRobertis, 1980). Adenosine 3':5'-cyclic monophosphate is small enough to pass through gap junctions from cell to cell, and it could presumably coordinate cellular activities in tissue regions, and hence establish fields (Hayes, 1976; Hoperskaya, 1976).

Cytochalasin B in the culture medium can stimulate a stellate configuration (Yamada, 1977). This compound is known to disrupt actin-containing microfilaments necessary for endocytosis (DeRobertis and

DeRobertis, 1980). Cells spread on a plastic surface may be regarded as being engaged in the futile attempt to engulf an object of infinitely large diameter. During mitosis endocytosis seems to be interrupted. Actin microfilaments increase in iris epithelial cells during lens regeneration (Dumont and Yamada, 1972), to form a network similar to the one induced by retinal extracts in cultured bovine lens epithelial cells (Courtois et al., 1981). Actin is also known to bind to deoxyribonuclease I and to inhibit its activity (Lazarides and Lindberg, 1974). This action may protect newly synthesized single-stranded DNA in newt iris cells (Collins, 1974a, b) during the synthesis phase of the cell cycle.

Epidermal growth factor also stimulates phosphorylation of proteins after attachment to its specific receptor on target cells (Cohen et al., 1979). The complex of growth factor and receptor is, however, rapidly internalized in endocytic vesicles and degraded after fusion of these vesicles with lysosomes (Cohen et al., 1979). This may very well also be the fate of the lens regeneration stimulating factor on responsive iris epithelial cells. In addition, the factor attached to its receptor could depolarize the plasmalemma and trigger contraction of actin-myosin microfilaments underlying that particular cell surface region (Courtois et al., 1981). This would lead to endocytosis, internalization and breakdown of the factor. The resulting dipeptides can be transported through the vesicular membrane and are cleaved into single amino acids by dipeptidases in the cytoplasm (DeRobertis and DeRobertis, 1980). Reese (1977) has suggested that intracellular increase of essential amino acids may control ribosomal ribonucleic acid synthesis in iris

Figure 58. Model of the action of the lens regeneration stimulating factor on iris epithelial cells. LRSF lens regeneration stimulating factor (a peptide or glycopeptide), LRSF-R receptor for LRSF, SP a generalized serum protein, AMP adenosine monophosphate, ADP adenosine diphosphate, ATP adenosine triphosphate, cAMP adenosine 3':5'-cyclic monophosphate, AC adenylate cyclase (LRSF-R dependent), PD phosphodiesterase, PPK protein phosphokinase (Ca^{2+}, and cAMP dependent), Ca^{2+} calcium ion, P protein-linked phosphate, T tubulin (polymerization PPK dependent), mp electrical membrane potential (+ positive, - negative), A-M actin-myosin microfilaments (contraction Ca^{2+} dependent), H lysosomal proteolytic hydrolases, LM lysosomal membrane, EVM endocytic vesicle membrane, CM cell membrane, NM nuclear membrane, NP nuclear pore, N nucleolus, DNA general chromosomal deoxyribonucleic acid, rDNA DNA coding for rRNA, rRNA ribosomal ribonucleic acid, mRNA messenger ribonucleic acid, R ribosome, n nucleotide, a amino acid, a-a dipeptide, DP dipeptidase. Accodring to this model, LRSF has a dual function. It increases endogenous cAMP, as proposed by Velázquez and Ortíz (1980), which stimulates polymerization of microtubules, as was suggested by the work of Ortíz et al. (1973) and Ortíz and Connelly (1977). The second function of LRSF is, according to this model, to induce actin-dependent (Courtois et al., 1981) endocytosis upon attachment to LRSF-R, as has been demonstrated in the case of the epidermal

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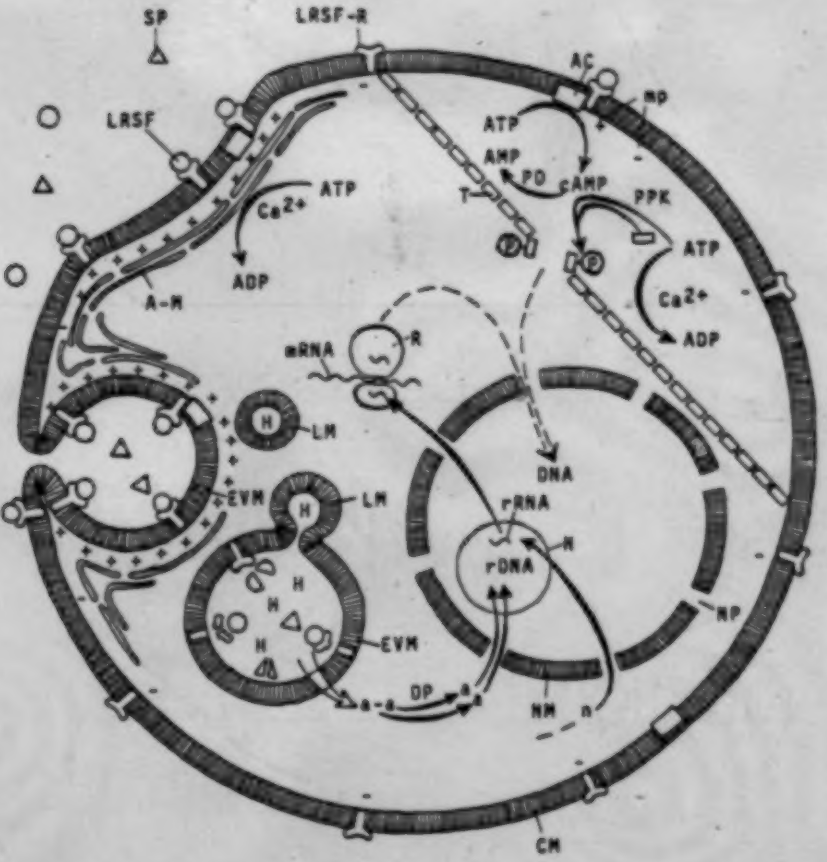
SP
LRSF-R
AC
mp
LRSF
ATP
AMP
PD
cAMP
T
PPK
ATP
Ca^{2+}
ADP
ATP
Ca^{2+}
ADP
A-M
mRNA
R
H
LM
DNA
EVM
H
LM
rRNA
N
rDNA
H
H
H
EVM
NP
a-a
DP
a
NM
n
CM

Figure 58. (...continued)

growth factor by Cohen et al. (1979). Hydrolysis of the peptides in the endocytic vesicles increases endogenous levels of essential amino acids, which directly stimulate rRNA transcription, as suggested by Reese (1977). How tubulin polymerization and ribosomal ribonucleic acid transcription eventually lead to DNA replication is not resolved by this model, nor is the switch in cell type explained.

epithelial cells of the newt, as is known for mammalian cells.

The above mentioned speculations imply that the lens regeneration stimulating factor acts on iris epithelial cells by changing simultaneously several intracellular synthetic pathways. This factor does not appear to trigger specific activation events, but rather seems to act as a trophic factor which must be present over a long time period. Organ cultures of irises with lens regenerates at different stages also demonstrated that lens regeneration stopped after a few days of separation of these irises from the lens regeneration stimulating factor in the eye (Zalik and Scott, 1969). If a pituitary gland is apposed to normal irises previously cultured in isolation for 5 days, lens regeneration still occurs, but at a lower frequency. However, if the pituitary is not added before day 10 or 15, the frequency of lens regeneration is no higher than in control cultures maintained in the absence of this gland. Only very few lenses formed if irises were cultured with pituitary glands only during the first 5 days of culture (Connelly, 1977b). All these data demonstrate that trophic stimulation of the regenerating lens is required during redifferentiation.

When iris epithelial cells have passed through at least six or more cell cycles (Yamada, 1977; Yamada and McDevitt, 1981), they may have transformed into lens cells. In the present experiments the first definite sign of this change in cell type in cell culture was noticed on the 25th day after explantation. At that time crystallin, likely β-crystallin (McDevitt and Brahma, 1979, 1981), was detected in some totally depigmented cells (Figure 35 a,b). These cells did not, however, contain γ-crystallin, a marker of young lens fiber cells (Figure 34).

Eguchi et al. (1974) also detected crystallin in few iris epithelial cells after 30 days of culture, and by 50 days 50% of the cell population was crystallin positive. Zalik and Dimitrov (1980) reported that 42 days of culture were required until the first crystallin-positive cells appeared. In the present work, after 50 days in culture, γ-crystallin was definitely present in a large number of depigmented cells (Figures 34, 35c,d). After several generations of subcultured iris cells of the TVI cell line (Reese et al., 1976), some cells still contained small amounts of α- and β-crystallin, but no γ-crystallin (McDevitt and Yamada, 1980). In the present experiments, γ-crystallin was lost in some high density cultures (Figure 34) by 110 days. It was still present in a small number of cells in the cultures innoculated at lower cell density (Figure 34). In some cases all crystallins may disappear already after 98 days of culture (Zalik and Dimitrov, 1980). TSH-B8 did not seem to influence the times of appearance and vanishing of crystallins (Figure 34, 35, 45). It is not known how crystallin disappears from a cultured cell population; it is possible that cells resorb it by autophagy (Yamada et al., 1978). Abe and Eguchi (1977) have mentioned that some cell degeneration occurs after 60 days of primary cell culture. During this study it became clear that some cell death occurred after 40 to 60 days of culture, and among these cells the majority seemed to be lens fiber cells, which contained γ-crystallin (Figure 35e,f). Such cells often contained large vacuoles, which presumably were telolysosomes, and no nuclei were visible. The impression emerges, that lens fiber cells do not survive very long in cell culture, and once they have undergone terminal differentiation with nuclear resorption, they loose their mobility and become very fragile. After every mitotic peak period, a new wave of iris epithelial cells transforms into lens cells, which later degenerate and die. Within a cultured iris epithelial cell population, the reservoir of convertible cells could be exhausted, because probably not all cells respond to serum factors or TSH-B8.

An event that sometimes starts about 5 days before the first mitotic cells become detectable, and continues throughout the culture period, is the clustering of the dorsal iris epithelial cells with the formation of round or elongated bodies. The onset of this activity may correlate with the release from cell cycle arrest in either G_0, G_1 or the beginning of the S-phase. TSH-B8-supplemented cultures generally had an increased amount of clusters (Figures 27, 28, 29, 37, 40, 43). This may be due to an enlarged proportion of mitotic cells, which give rise to a faster growing cell population. Although generally mitotic peak periods are followed by periods of enhanced clustering, there seem to be peaks of clustering of iridal cells, which are not correlated with mitotic activity (Figure 27). Iris epithelial cell clusters can dissociate into smaller clusters, or aggregate into larger ones. Peak periods of frequency of iridal bodies cannot be explained by dissociation of large bodies into many smaller ones, because during the peak period iridal bodies also reach their maximal sizes. It is more likely, that iris epithelial cells in lammelar configuration acquire a stellate configuration and join to form iridal bodies.

Unlike iridal bodies, lentoid bodies only formed after the cells had passed several mitotic cell cycles, had lost their pigmentation and had accumulated at least β-crystallin. This occurred after 20 (Figures 38, 41, 44) to 25 days (Figures 27, 28, 29). TSH-B8 did not decrease the time necessary for the appearance of lentoid bodies, suggesting that this preparation had little if any influence on the cell cycle time. The first lentoid bodies were small, composed of only about 5 to 30 cells, but were compact and often spherical in outline. They clearly rose above the level of the cell sheet of lamellar cells. Mitotically dividing cells are abundant in these early lentoid bodies (Zalik and Dimitrov, 1980), and in the present experiments cells did not stop proliferating as they formed three-dimensional aggregates, contrary to the report of Abe and Eguchi (1977). Although a portion of these early lentoid bodies was probably formed by a clone derived from a single iris epithelial cell, other bodies were produced by progenies of several different parental cells. Some lentoid bodies have been reported to contain various amounts of totally pigmented cells (Zalik and Dimitrov, 1980). Although in agreement with Eguchi et al. (1974),in high cell density cultures (Figure 27) lentoid bodies emerge just before the cell population reaches saturation density, my experiments show that at lower cell density lentoid bodies can form in the middle of the logarithmic growth phase (Figures 28, 29, 38, 41, 44). The first generation of lentoid bodies differentiates terminally, becomes cataractous, with large vacuoles,and eventually dies between day 40 and 60 of culture. A second generation of lentoid bodies subsequently forms, mainly from partially to totally depigmented iridal bodies. Unlike that of iridal bodies, the frequency of lentoid bodies seems to directly depend on mitotic activity (Figure 28). The second generation of lentoid bodies yields the largest ones, which are sometimes linked to one another. TSH-B8 may slightly increase the size of iridal and lentoid bodies. The next

Figure 59. Cell type conversion and formation of iridal and lentoid bodies; hr hours, d days; black totally pigmented, dotted partially depigmented, white totally depigmented, and possibly crystallin-positive cells. Nuclei are first small, become enlarged, pass through mitosis, indicated by a pair of symbolized chromosomes, and eventually become small again. Cell shape changes from spherical during innoculation, 0 hr, to lamellar after 2 hr to 10 days in attached cells. In preparation for mitosis, which begins after 10 to 15 days of cultivation, cells assume spherical stellate configuration. Depigmentation starts 5 to 25 days after explantation. Repigmentation also seems to occur frequently, but this is not shown on the diagram. Iridal bodies emerge after 5 to 25 days. The lentoid bodies formed from 20 to 40 days onwards are mostly derived from single migrating or small clonal clusters of β-crystallin-containing cells. The second wave of lentoid bodies mainly stems from depigmented iridal bodies, and emerges about from days 35 to 50 onwards. At this time, γ-crystallin is present in most clusters. During the third and fourth wave of lentoid body production, from day 60 on, lentoid bodies frequently form domains or margins on larger iridal bodies, which are now composed of repressed iris epithelial cells incapable of changing their cell type, degenerating lentoid cells, and possibly some stromal cells. Degenerating lentoid bodies of cataractous appearance, still containing γ-crystallin,

(continued...)

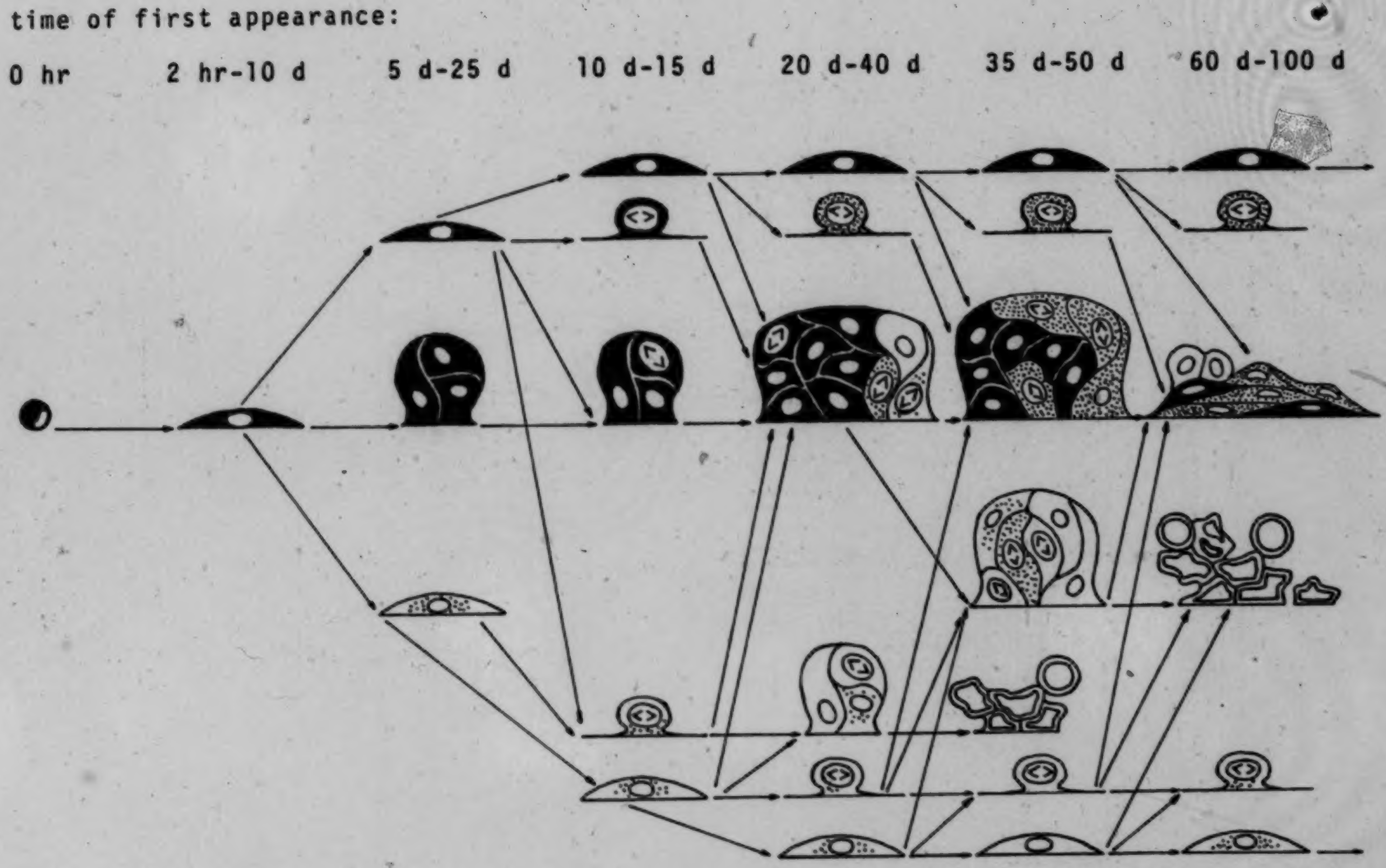
time of first appearance:
0 hr
2 hr-10 d
5 d-25 d
10 d-15 d
20 d-40 d
35 d-50 d
60 d-100 d

Figure 59. (...continued)

are seen from about day 40 on. They are composed of mostly terminally differentiated lentoid cells, which are irregularly shaped, and partially associated. These cells contain large vacuoles, which could possibly be telolysosomes, as shown in the diagram. At every stage, some single cells may join or leave the clusters. Also, the frequent dissociation of clusters into two or more smaller clusters is not depicted.

two generations of lentoid bodies are made up of freshly recruited lens cells either derived from iridal bodies or from sheets of lamellar cells. They usually form a domain or margin on an aged, fuzzy, and irregularly shaped iridal body, and one gains the impression that the source of convertible cells into lentoid cells is being exhausted. However, even in clusters classified as iridal bodies, a large portion of cells still may contain γ-crystallin (Figure 35g,h).

In high cell density cultures, proportionally more iridal bodies than lentoid bodies are found (Figure 27), when compared to low density cultures (Figures 28, 29), probably because saturation density is reached before iris epithelial cells have passed sufficient cell cycles to convert into lens cells. Also, the cell density in a single dish is usually higher in the center, due to the passive floating of freshly innoculated unattached cells in the culture medium. As can now be predicted, iridal bodies preferably form in the central region with the highest cell density, and lentoid bodies, which require more mitotic activity form more peripherally. In cell cultures innoculated at very low cell density, lentoid and iridal bodies never reached a comparable frequency, when compared to medium cell density cultures, although in this case eventually the cell population increases to medium and high cell density levels. It seems that the proportion of originally seeded responsive iris epithelial cells per iris is limited, and these cells are lost in dying lentoid bodies, before a higher cell density is reached.

In summary, TSH-B8 can stimulate an increased portion of iris epithelial cells to pass through mitotic cell cycles which may lead to cell type conversion. It also enhances the proportion of cluster-forming iris epithelial cells or lentoid cells. During dedifferentiation in the first 2 to 4 days of culture, cells do not seem to be responsive to TSH-B8; later, during redifferentiation, TSH-B8 must be continuously present to support the conversion of an increased number of iris epithelial cells into lens cells. Depigmentation is not directly controlled by TSH-B8. Although the present data are not sufficient, they do not seem to support the idea that TSH-B8 shortens the cell cycle time.

6. RESPONSE OF THE NEURAL RETINA TO LENS REMOVAL

For lentectomy the eye must be opened and the retina and iris become exposed to a drop in pressure of ocular fluids. This may result in an increase in serum ultrafiltration from the choroid by retinal pigmented epithelial cells and back-flow of lymphatic humour from the Schlemm canal (Reinke, 1906; Rapoport, 1977). It seems that the basement membrane of the internal lamina of the iris epithelium is altered after the removal of the lens in the newt (*Notophthalmus viridescens*)(Dumont and Yamada, 1972), the salamander (*Salamandra salamandra*), and the frog (*Rana pipiens*)(Uhlenhut, 1919). This could be a consequence of the swelling of iris epithelial cells, and shedding of pigment granules (Uhlenhut, 1919). The more direct exposure of the iris epithelium to serum components and possibly to lens regeneration stimulating factor from the retina may change its cell program (Uhlenhut, 1919; Yamada, 1977). Also, removal of extracellular matrix material could change their responsiveness to growth factors. Epidermal growth factor only acts on corneal endothelial cells as long as they are in contact with extracellular matrices (Gospodarowicz et al., 1979, 1980). Cultured pigmented retinal cells do not transdifferentiate when grown on collagen (Eguchi, 1979). In the process of lens regeneration, extracellular matrix material (Kulyk and Zalik, 1979) is restored by the iris.

The inner limiting basement membrane of the neural retina of the frog (*Rana pipiens*) is also shed after lentectomy (Figure 53). This may greatly increase flow of vitreous humour ultrafiltrate through the pigmented retina into the vitreous chamber. Indirect evidence for this assumption was provided by autoradiography of eyes utilizing ^{3}H-leucine as protein precursor (Gulati and Reyer, 1980), which showed that the protein concentration within the vitreous humour is increased after lens removal. In the present studies, the shed inner limiting basement membrane was lying in the vitreous chamber 5 days after lentectomy, still staining with the fluorescein-labelled antibody. Mikami (1941b) noticed that lens regeneration may be retarded in iris pieces grafted close to the retina. It could be that such grafts became wrapped up in the shed basement membrane for the first few days, which possibly decreased their accessibility for the lens regeneration stimulating factor.

The first reported reaction of the neural retina to lens removal is an increase in ribonucleic acid synthesis (Gulati, 1980). This change occurs on day 0, and reaches a maximum on day 3 after lentectomy, to slowly decrease later. Protein synthesis, measured by ^{3}H-leucine incorporation (Gulati, 1980), is depressed until day 2, but is elevated from then on until day 18, similar to the pattern of endogenous adenosine 3':5'-cyclic monophosphate levels (Velázquez and Ortíz, 1980). However, with the less sensitive autoradiography method, no differences of protein synthesis before and after lentectomy could be visualized (Gulati and Reyer, 1980). The incorporation of ^{3}H-fucose possibly into glycoproteins throughout the neural retina is enhanced by day 12 (Reyer and Song, 1981). In the region of the ora serrata of the neural retina mitotic activity does not seem to appear until 20 days after lentectomy (Yamada and Roesel, 1971). When the retina is more severely damaged, retinal regeneration may be under way earlier, also in the central region of the retina (Keefe, 1973b). During total retinal regeneration, Müller and ganglion cells differentiate first (Keefe, 1973b). Since the retina can stimulate lens regeneration early during its own regeneration (Zalokar, 1944; Stone and Steinitz, 1953a) the pigment retina cells, the Müller cells or the ganglion cells could be the likely sources of the lens regeneration stimulating factor.

After 5 days of lens regeneration, the inner processes of the Müller cells contain a material which cross-reacts with antiserum directed against crude pituitary glycoprotein extract. It is known, that the Müller cells produce the inner limiting basement membrane (Bloom and Fawcett, 1975), and it is very likely that the cross-reacting material is later deposited in the inner limiting basement membrane. This restoration of the inner limiting basement membrane is observed in the frog. After 14 days, the inner limiting basement membrane is fully regenerated (Figure 53). Although the iris of the frog does not have the potential to form a lens, an implanted newt iris can regenerate a lens in the eye of a nonregenerating species.

In the present experiments the antiserum prepared against a pituitary glycoprotein reacted with 3 to 6 major components of the pituitary extract (Figure 52). Most cells and the collagenous capsule of the adenohypophysis were positive for these antigens as shown by immunofluorescence. Not all basement membranes in the eye were positive for this antigen. Exceptions were Bruch's basement membrane of the pigment retina, and Bowman's basement membrane of the corneal epithelium. There is a good possibility that this antiserum may detect collagen, especially if glycosylated. In bovine eyes, vitreous body collagen is synthesized by retinal cells and hyalocytes (Newsome et al., 1975). Macromolecules in the newt vitreous chamber are also at least partially derived from the neural retina (Fischel, 1900).

Choroidal serum and vitreous chamber fluid cross-reacted weakly with the antiserum. Fibronectin is an extracellular matrix glycoprotein, which also occurs in a cold-insoluble serum form. In the embryonic chick retina, it is present in the inner limiting basement membrane (Kurkinen et al., 1979). Also, during stimulation with retinal extract, bovine lens epithelial cells produce fibronectin (Courtois et al., 1981). In the present work, using a ring test, no antibodies against human cold-insoluble fibronectin could be detected in the anti-frog pituitary crude glycoprotein extract antiserum. Because of the species specificity of antibody reactions, this result may not be very decisive, however. It is still possible that frog fibronectin is detected by the antibody. In addition to basement membrane material, Müller cells could also produce lens regeneration stimulating factor. The experiment described above indicates that at least one antigen is produced by the Müller cells after lentectomy, which cross-reacts with anti-pituitary crude glycoprotein antiserum. The significance of this observation remains to be established.

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